

FUSION MOLECULES AND TREATMENT OF IgE-MEDIATED ALLERGIC DISEASESBackground of the Invention

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Field of the Invention

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The invention concerns a new approach for the management of IgE-mediated allergic diseases and other disorders mediated through IgE receptors (FcεRs) using novel fusion molecules that are able to complex with an FcεR and an inhibitory receptor expressed on mast cells, basophils, or B cells, including inhibitory receptors having an immune receptor tyrosine-based inhibitory (ITIM) motif.

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Description of the Related Art

Immunoglobulin receptors (also referred to as Fc receptors) are cell-surface receptors binding the constant region of immunoglobulins, and mediate various immunoglobulin functions other than antigen binding.

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Fc receptors for IgE molecules are found on many cell types of the immune system (Fridman, W., *FASEB J.*, 5(12):2684-90 (1991)). There are two different receptors currently known for IgE. IgE mediates its biological responses as an antibody through the multichain high-affinity receptor, FcεRI, and the low-affinity receptor, FcεRII. The high-affinity FcεRI, expressed on the surface of mast cells, basophils, and Langerhans cells, belongs to the immunoglobulin gene superfamily, and has a tetrameric structure composed of an α-chain, a β-chain and two disulfide-linked γ-chains (Adamczewski, M., and Kinet, J.P., *Chemical Immun.*, 59:173-190 (1994)) that are required for receptor expression and signal transduction (Tunon de Lara, *Rev. Mal. Respir.*, 13(1):27-36 (1996)). The α-chain of the receptor interacts with the distal portion of the third constant domain of the IgE heavy chain. The specific amino acids of human IgE involved in binding to human FcεRI have been identified as including Arg-408, Ser-411, Lys-415, Glu-452,

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Arg-465, and Met-469 (Presta *et al.*, *J. Biol. Chem.* 269:26368-73 (1994)). The interaction is highly specific with a binding constant of about 10^{10} M^{-1} .

The low-affinity FcεRII receptor, represented on the surface of inflammatory cells, including eosinophils, leukocytes, B lymphocytes, and platelets, did not evolve from the immunoglobulin superfamily but has substantial homology with several animal lectins (Yodoi *et al.*, *Ciba Found. Symp.*, 147:133-148 (1989)) and is made up of a transmembrane chain with an intracytoplasmic NH2 terminus. The low-affinity receptor, FcεRII (CD23) is currently known to have two forms (FcεRIIa and FcεRIIb), both of which have been cloned and sequenced. They differ only in the N-terminal cytoplasmic region, the extracellular domains being identical. FcεRIIa is normally expressed on B cells, while FcεRIIb is expressed on T cells, B cells, monocytes and eosinophils upon induction by the cytokine IL-4.

Through the high-affinity IgE receptor, FcεRI, IgE plays key roles in an array of acute and chronic allergic reactions, including asthma, allergic rhinitis, atopic dermatitis, severe food allergies, chronic urticaria and angioedema, as well as the serious physiological condition of anaphylactic shock as results, for example, from bee stings or penicillin allergy. Binding of a multivalent antigen (allergen) to antigen specifically bound to FcεRI on the surface of mast cells and basophils stimulates a complex series of signaling events that culminate in the release of host vasoactive and proinflammatory mediators contributing to both acute and late-phase allergic responses (Metcalf *et al.*, *Physiol. Rev.* 77:1033-1079 (1997)).

The function of the low affinity IgE receptor, FcεRII (also referred to as CD23), found on the surface of B lymphocytes, is much less well established than that of FcεRI. FcεRII, in a polymeric state, binds IgE, and this binding may play a role in controlling the type (class) of antibody produced by B cells.

Three groups of receptors that bind the constant region of human IgG have so far been identified on cell surfaces: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), all of which belong to the immunoglobulin gene superfamily. The three Fcγ receptors have a large number of various isoforms.

Along with the stimulatory FcεRI, mast cells and basophils co-express an immunoreceptor tyrosine-based inhibition motif (ITIM)-containing inhibitory low-affinity receptor, FcγRIIb, that acts as a negative regulator of antibody function. FcγRIIb represents a growing family of structurally and functionally similar inhibitory receptors, the inhibitory receptor superfamily (IRS),

that negatively regulate ITAM-containing immune receptors (Ott and Cambier, *J. Allergy Clin. Immunol.*, 106:429-440 (2000)) and a diverse array of cellular responses. Coaggregation of an IRS member with an activating receptor leads to phosphorylation of the characteristic ITIM tyrosine and subsequent recruitment of the SH2 domain-containing protein tyrosine phosphatases, SHP-1 and SHP-2, and the SH2 domain-containing phospholipases, SHIP and SHIP2 (Cambier, J.C., *Proc. Natl. Acad. Sci. USA*, 94:5993-5995 (1997)). Possible outcomes of the coaggregation include inhibition of cellular activation, as demonstrated by the coaggregation of FcγRIIb and B-cell receptors, T-cell receptors, activating receptors, including FcεRI, or cytokine receptors (Malbec et al., *Curr. Top. Microbiol. Immunol.*, 244:13-27 (1999)).

Most studies have so far concentrated on elucidating the mechanisms of FcγRII, in particular FcγRIIb, function. The three alternatively spliced isoforms of the FcγRIIb receptor, of which FcγRIIb1' is only found in mice, and FcγRIIb1 and FcγRIIb2 are expressed in both humans and mice, have Ig-like loops and a conserved ITIM, but differ in their cytoplasmic domains. Co-crosslinking of the high-affinity FcεRI receptor and the inhibitory low-affinity receptor FcγRII blocks a number of processes, including FcεRI-mediated secretion, IL-4 production, Ca²⁺ mobilization, Syk phosphorylation, and FcεRI-mediated basophil and mast cell activation. In B cells, co-crosslinking of the B-cell receptor and FcγRIIb inhibits B-cell receptor-mediated cell activation (Cambier, J.C., *Proc. Natl. Acad. Sci.*, 94:5993-5995 (1997); Daeron, M., *Annu. Rev. Immunol.*, 5:203-234 (1997)), and specifically, inhibits B-cell receptor-induced blastogenesis and proliferation (Chan et al., *Immunology*, 21:967-981 (1971); Phillips and Parker, *J. Immunol.*, 132:627-632 (1984)) and stimulates apoptosis (Ashman et al., *J. Immunol.*, 157:5-11 (1996)). Coaggregation of FcγRIIb1 or FcγRIIb2 with FcεRI in rat basophilic leukemia cells, inhibits FcεRI-mediated release of serotonin and TNF-α (Daeron et al., *J. Clin. Invest.*, 95:577-85 (1995); Daeron et al., *Immunity*, 3:635-646 (1995)).

Another ITIM-containing receptor expressed on mast cells that has been described to prevent IgE-mediated mast cell activation when coligated with FcεRI, is a 49 kDa glycoprotein member of the immunoglobulin superfamily, termed gp49b1 (gp91) (see, e.g. Wagtmann et al., *Current Top. Microbiol. Immunol.* 244:107-113 (1999); Katz, H.R., *Int. Arch Allergy Immunol.* 118:177-179 (1999); and Lu-Kuo et al., *J. Biol. Chem.* 274:5791-96 (1999)). Gp49b1 was originally identified in mice, while human counterparts of the gp49 family, including gp49b1, have been cloned by Arm

et al., *J. Immunol.* 15:2342-2349 (1997). Further ITIM-containing receptors, several expressed in mast cells, basophils or B cells are reviewed by Sinclair NR, *Scand. J. Immunol.* 50:10-13 (1999).

Despite advances in understanding the cellular and molecular mechanisms that control allergic responses and improved therapies, the incidence of allergic diseases, especially asthma, has increased dramatically in recent years in both developed and developing countries (Beasley et al., *J. Allergy Clin. Immunol.* 105:466-472 (2000); Peat and Li, *J. Allergy Clin. Immunol.* 103:1-10 (1999). Allergic diseases can be treated, for example, by allergen-based vaccination, in which increasing doses of allergen are given by injection over years. This approach is costly, time consuming, poorly or not efficacious in many allergic conditions, and has serious side-effects, including death in some instances. Mild asthma can usually be controlled in most patients by relatively low doses of inhaled corticosteroids, while moderate asthma is usually managed by the additional administration of inhaled long-acting β -antagonists or leukotriene inhibitors. The treatment of severe asthma is still a serious medical problem. In addition, many of the therapeutics currently used in allergy treatment have serious side-effects. Although an anti-IgE antibody currently in clinical trials (rhuMAb-E25, Genentech, Inc.) and other experimental therapies (e.g. antagonists of IL-4) show promising results, there is need for the development of additional therapeutic strategies and agents to control allergic disease, such as asthma, severe food allergy, and chronic urticaria and angioedema.

The object of this invention is to provide a novel therapeutic strategy designed to cross-link inhibitory receptors expressed on mast cells, basophils and/or B cells, such as an ITIM-containing Fc γ RIIb or gp49b1 receptor, or p91/PIR-B receptor, with Fc ϵ RI or Fc ϵ RII, for the treatment of conditions associated with anaphylactic hypersensitivity and atopic allergies, such as, for example, asthma, allergic rhinitis, atopic dermatitis, severe food allergies, some forms of chronic urticaria and angioedema, as well as the serious physiological condition of anaphylactic shock as results, for example, from bee stings or penicillin allergy.

Summary of the Invention

The present invention provides novel bi-functional compounds that co-crosslink inhibitory receptors with Fc ϵ receptors and block Fc ϵ receptor-mediated biological activities, as well as methods of making and using such compounds, and compositions and articles of manufacture comprising them.

In one aspect the invention concerns an isolated fusion molecule comprising a first polypeptide sequence capable of specific binding, to a native inhibitory receptor comprising an immune receptor tyrosine-based inhibitory motif (ITIM), expressed on mast cells, basophils and/or B cells, functionally connected to a second polypeptide sequence capable of specific binding, directly or indirectly, to a native IgE receptor (FcεR). Preferably, the inhibitory receptor is a type I transmembrane molecule with an Ig-like domain, such as, for example, a low-affinity IgG receptor FcγRIIb, an inhibitory receptor of the gp49 family, e.g. gp49b1, p91/PIR-B, leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1), LIR-1, or CD22.

The IgE receptor may be a high-affinity FcεRI receptor, or a low-affinity FcεRII receptor (CD23).

More preferably, the inhibitory receptor is a low-affinity FcγRIIb receptor, most preferably native human FcγRIIb, and the IgE receptor is a high-affinity FcεRI receptor, most preferably native human FcεRI, although fusion molecules including sequences capable of specific binding, directly or indirectly, to the low-affinity IgE receptor FcεRII are also within the scope of the invention.

In a particularly preferred embodiment, the two receptors are both of human origin, and the first and second polypeptide sequences present in the fusion molecules are human IgG, e.g. IgG₁, and IgE heavy chain constant region sequences, respectively.

In a preferred embodiment, the second polypeptide sequence comprises a sequence of an allergen protein, which is capable of indirect binding to a high- or low-affinity IgE receptor via an allergen-specific IgE molecule. In this embodiment, the second polypeptide sequence may comprise part or whole of a native or variant allergen protein, such as a food or pollen allergen.

The first and second polypeptide sequences may be connected via a linker, e.g. a polypeptide linker or a non-polypeptide bifunctional linker, or may be directly fused to each other. The length of the polypeptide linker typically is about 5 to 25 amino acid residues, preferably about 10 to 25 amino acid residues, most preferably about 15 to 25 amino acid residues.

In a particular embodiment, the first polypeptide sequence in the fusion molecule retains the residues from a native IgG heavy chain constant region that are required to bind to the targeted IgG inhibitory receptor, e.g. FcγRIIb. Similarly, in a particular embodiment, the second polypeptide sequence in the fusion molecule retains the residues from a native IgE heavy chain constant region that are required for binding to the targeted IgE receptor, such as FcεRI or FcεRII.

In another embodiment, the first polypeptide sequence comprises an amino acid sequence having at least about 80%, preferably at least about 85%, more preferably at least about 90%, even more preferably at least about 95%, most preferably at least about 99% sequence identity with the hinge-CH2-CH3 portion of a native IgG immunoglobulin heavy chain constant region. The IgG preferably is, but does not need to be, IgG₁. Indeed, the IgG portion of the molecule can derive from the heavy chain constant region of any IgG subclass, including IgG₁, IgG₂, IgG₃ and IgG₄.

In yet another embodiment, the first polypeptide sequence comprises an amino acid sequence having at least about 80%, preferably at least about 85%, more preferably at least about 90%, even more preferably at least about 95%, most preferably at least about 99% sequence identity with the receptor-binding domain of a ligand of another native ITIM-containing inhibitory receptor expressed on mast cells, basophils, or B cells, such as, without limitation, a native gp49b1, p91/PIR-B, LAIR-1, LIR-1, or CD11 receptor.

In another particular embodiment, the second polypeptide sequence in the fusion molecule comprises an amino acid sequence having at least about 80%, preferably at least about 85%, more preferably at least about 90%, even more preferably at least about 95%, most preferably at least about 99% sequence identity with the CH2-CH3-CH4 portion of a native IgE immunoglobulin heavy chain constant region.

In yet another embodiment, the second polypeptide sequence in the fusion molecule comprises an amino acid sequence having at least about 80%, preferably at least about 85%, more preferably at least about 90%, even more preferably at least about 95%, most preferably at least about 99% sequence identity with a native allergen protein or a fragment thereof.

In a further embodiment, the first polypeptide sequence in the fusion molecule comprises an amino acid sequence encoded by nucleic acid hybridizing under stringent conditions to the complement of the coding sequence of the hinge-CH2-CH3 portion of a native IgG immunoglobulin heavy chain constant region, and retains the ability to bind an IgG inhibitory receptor, preferably human FcγRIIb. The IgG preferably is, but does not need to be, IgG₁.

In a still further preferred embodiment, the second polypeptide sequence in the fusion molecule comprises an amino acid sequence encoded by nucleic acid hybridizing under stringent conditions to the complement of the coding sequence of the CH2-CH3-CH4 portion of a native IgE immunoglobulin heavy chain constant region, and retains the ability to bind a high-affinity IgE receptor, preferably human FcεRI.

In yet another embodiment, the second polypeptide sequence in the fusion molecule comprises an amino acid sequence encoded by nucleic acid hybridizing under stringent conditions to the complement of the coding sequence of all or part of a native allergen protein.

5 A particularly preferred molecule of the invention comprises the hinge-CH2-CH3 portion of an IgG, such as IgG₁, immunoglobulin heavy chain constant region functionally linked to the CH2-CH3-CH4 portion of an IgE immunoglobulin heavy chain constant region via a 15 amino acids polypeptide linker. In a preferred embodiment, the IgG₁ hinge-CH2-CH3 sequence is connected at its C-terminus to the N-terminus of the IgE CH2-CH3-CH4 sequence via the 15 amino acids polypeptide linker. Preferably both immunoglobulin heavy chain sequences are of human origin.

10 In another aspect, the invention concerns isolated nucleic acid molecules encoding polypeptide fusions of the present invention. The invention also concerns vectors comprising such nucleic acid molecules, and recombinant host cells transformed with such vectors.

15 In a further aspect, the invention concerns a pharmaceutical composition comprising a fusion molecule as hereinabove defined in admixture with a pharmaceutically acceptable ingredient. The pharmaceutical composition is preferably used for the treatment of an IgE-mediated biological response, such as an acute or late phase allergic reaction, including, without limitation, immediate hypersensitivity reactions. In a preferred embodiment, the pharmaceutical composition is for the treatment of a condition associated with anaphylactic hypersensitivity or an atopic allergy, such as asthma, allergic rhinitis, atopic dermatitis, severe food allergies, chronic urticaria, angioedema, and/or anaphylactic shock.

20 In a still further aspect, the invention concerns an article of manufacture comprising a container, a fusion molecule as hereinabove defined within the container, and a label or package insert on or associated with the container. The label or package insert preferably comprises instructions for the treatment of a condition associated with an IgE-mediated biological response, such as a condition associated with anaphylactic hypersensitivity or an atopic allergy, e.g. asthma, allergic rhinitis, atopic dermatitis, severe food allergies, chronic urticaria, angioedema, and/or anaphylactic shock.

25 In yet another aspect, the invention concerns a method for the treatment of a condition associated with an IgE-mediated biological response, comprising administering an effective amount of a fusion molecule as hereinabove defined to a subject in need. The subject preferably is a human

patient, and the condition to be treated (including prevention), preferably is asthma, allergic rhinitis, atopic dermatitis, severe food allergies, chronic urticaria, angioedema, and/or anaphylactic shock.

In addition to the aspects discussed above, the present invention also contemplates fusion molecules suitable for coaggregation of other inhibitory receptors with IgE receptors, such as FcεRI or FcεRII. For example, a fusion molecule comprising a c-kit ligand sequence, capable of specific binding the receptor PTK c-Kit, fused to a polypeptide sequence capable of specific binding, directly or indirectly, to an IgE receptor, such as FcεRI or FcεRII, is also contemplated. Such fusion molecules are expected to negatively regulate the expression of mast cells, and find utility in the treatment of conditions associated with anaphylactic hypersensitivity and atopic allergies.

Brief Description of the Figures

Figure 1 shows the nucleotide sequence encoding the human IgG₁ heavy chain constant region (SEQ ID NO: 1).

Figure 2 shows the amino acid sequence of the human IgG₁ heavy chain constant region (SEQ ID NO: 2). In the sequence, the CH1 domain extends from amino acid position 122 to amino acid position 219, the hinge region extends from amino acid position 220 to amino acid position 231, the CH2 domain extends from amino acid position 232 to amino acid position 344, and the CH3 domain extends from amino acid position 345 to amino acid 451 (the C-terminus).

Figure 3 shows the amino acid sequence of the hinge-CH2-CH3 portion of the human IgG₁ heavy chain constant region (SEQ ID NO: 3).

Figure 4 shows the nucleotide sequence encoding the human IgE heavy chain constant region (SEQ ID NO: 4).

Figure 5 shows the amino acid sequence of the human IgE heavy chain constant region (SEQ ID NO: 5).

Figure 6 shows the amino acid sequence of the CH2-CH3-CH4 portion of the human IgE heavy chain constant region (SEQ ID NO: 6).

Figure 7 shows the amino acid sequence of the γhinge-CHγ2-CHγ3-(Gly₄Ser)₃-CHε2-CHε3-CHε3 fusion molecule (GE2) of the invention (SEQ ID NO: 7).

Figure 8 illustrates the dose-dependent inhibition of basophil histamine release using the fusion protein GE2 (± SEM; n=3 separate donors, each in duplicate). Purified human blood basophils were acid stripped and then sensitized with humanized anti-NP IgE, labeled as IgE, alone

or in the presence of GE2 protein or PS that is a purified human IgE myeloma protein. One hour later, cells were challenged with NP-BSA and the resulting level of histamine release measured.

Figure 9 shows results obtained in the transgenic passive cutaneous anaphylaxis (PCA) model described in the Example. Sites were injected with 250 ng of human anti-IgE NP along with the indicated amounts of PS (non-specific human IgE) or GE2 chimeric fusion protein. Four hours later, the animals were challenged intravenously (IV) with 500 µg of NP-BSA.

Figure 10 illustrates GE2 binding to HMC-1 cells that express FcγRIIb but not FcεRIa.

Figure 11 illustrates GE2 binding to 3D10 cells that express FcεRIa but not FcγRIIb.

Detailed Description of the Preferred Embodiment

I. Definitions

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Indeed, the present invention is in no way limited to the methods and materials described. For purposes of the present invention, the following terms are defined below.

The term “functionally connected” with reference to the first and second polypeptide sequences included in the fusion molecules herein, is used to indicate that such first and second polypeptide sequences retain the ability to bind to the respective receptors. Thus, after being connected to a second polypeptide sequence, the first polypeptide sequence retains the ability of specific binding to a native IgG inhibitory receptor, such as a low-affinity FcγRIIb receptor. Similarly, after being connected to a first polypeptide sequence, the second polypeptide sequence retains the ability of specific binding, directly or indirectly, i.e. through a third polypeptide sequence, to a native IgE receptor, such as a native high-affinity IgE receptor, e.g. native human FcεRI, or a native low-affinity IgE receptor, e.g. FcεRII. As a result, the fusion molecule, comprising the first and second polypeptide sequences functionally connected to each other, is capable of cross-linking the respective native receptors, such as, for example, FcγRIIb and FcεRI or FcεRII. In order to achieve a functional connection between the two binding sequences within the fusion molecules of the invention, it is preferred that they retain the ability to bind to the

corresponding receptor with a binding affinity similar to that of a native immunoglobulin heavy chain or other native polypeptide binding to that receptor.

The binding is "specific" when the binding affinity of a molecule for a binding target, e.g. an IgG or IgE receptor, is significantly higher (preferably at least about 2-times, more preferably at least about 4-times, most preferably at least about 6-times higher) than the binding affinity of that molecule to any other known native polypeptide. Since you do not define how one determines the universe of "other known native polypeptide(s)", this definition could be considered indefinite. What about defining specific binding as preferential binding in the presence of a competitor (you could even name possible competitors).

The term "inhibitory receptor" is used in the broadest sense and refers to a receptor capable of down-regulating a biological response mediated by another receptor, regardless of the mechanism by which the down-regulation occurs.

The terms "receptor comprising an immune receptor tyrosine-based inhibitory motif (ITIM)" and "ITIM-containing receptor" is used to refer to a receptor containing one or more immune receptor tyrosine-based inhibitory motifs, ITIMs. The ITIM motif can be generally represented by the formula Val/Ile-Xaa-PTyr-Xaa-Xaa-Leu/Val (where Xaa represents any amino acid). ITIM-containing receptors include, without limitation, FcγRIIb, gp49b1/gp91 (Arm *et al.*, *J. Biol. Chem.* 266:15966-73 (1991)), p91/PIR-B (Hayami *et al.*, *J. Biol. Chem.* 272:7320-7 (1997)), LIR1-3, 5, 8, LAIR-1; CD22 (van Rossenberg *et al.*, *J. Biol. Chem.* January 4, 2001); CTL-4, CD5, p58/70/140 KIR, PIRB2-5; NKB1, Ly49 A/C/E/F/G, NKG2-A/B, APC-R, CD66, CD72, PD-1, SHPS-1, SIRP-α1, IL T1-5, MIR7, 10, hMIR(HM18), hMIR(HM9), Fas(CD95), TGFβ-R, TNF-R1, IFN-γ-R (α- and β-chains), mast cell function Ag, H2-M, HLA-DM, CD1, CD1-d, CD46, c-cbl, Pyk2/FADK2, P130 Ca rel prot, PGDF-R, LIF, LIR-R, CIS, SOCS13 and 3, as reviewed in Sinclair NR *et al.*, *supra*. Ligands for many of these receptors are also known, such as, e.g. the ligand for CD95 is called CD95 ligand, the ligands for CTLA-4 are CD80 and CD86, the ligands of IFN-γ receptor is IFN-γ, etc. Ligands for CD22 comprise the basic binding motif Nau5Ac-a(2,6)-Lac, and are discussed, for example in van Rossenberg *et al.*, 2001, *supra*.

The term "IgG inhibitory receptor" is used to define a member of the inhibitory receptor superfamily (IRS), now known or hereinafter discovered, that is capable of attenuating an FcεR-mediated response, regardless of whether it is mediated via IgE acting through a high-affinity IgE receptor, e.g. FcεRI, or a low-affinity IgE receptor, or by another mechanism such as an

autoantibody to the FcεR. The response preferably is an IgE-mediated allergic response, such as a type I (immediate hypersensitivity) reaction but could include autoimmune reactions due to anti-FcεRI α-chain antibodies that have been reported in about half of the cases of chronic idiopathic urticaria.

5 The term “native” or “native sequence” refers to a polypeptide having the same amino acid sequence as a polypeptide that occurs in nature. A polypeptide is considered to be “native” in accordance with the present invention regardless of its mode of preparation. Thus, such native sequence polypeptide can be isolated from nature or can be produced by recombinant and/or synthetic means. The terms “native” and “native sequence” specifically encompass naturally-
10 occurring truncated or secreted forms (*e.g.*, an extracellular domain sequence), naturally-occurring variant forms (*e.g.*, alternatively spliced forms) and naturally-occurring allelic variants of a polypeptide.

 The terms “native FcγRIIb,” “native sequence FcγRIIb,” “native low-affinity IgG inhibitory receptor FcγRIIb,” and “native sequence low-affinity IgG inhibitory receptor FcγRIIb” are used interchangeably, and refer to FcγRIIb receptors of any species, including any mammalian species, as
15 occurring in nature. Preferably, the mammal is human. FcγRIIb is an isoform of the low-affinity IgG receptor FcγRII containing an immunoreceptor tyrosine-based inhibition motif (ITIM). This receptor is the principal FcγRII species in human peripheral blood basophils and cord blood-derived mast cells. For further details see, for example, Malbec and Fridman, *Curr. Top. Microbiol. Immunol.* 244:13-27 (1999); Cambier, J.C., *Proc. Natl. Acad. Sci. USA* 94:5993-5995 (1997); and
20 Ott and Cambier, *J. Allergy Clin. Immunol.* 106(3):429-440 (2000). FcγRIIb has three alternatively spliced forms designated FcγRIIb1, FcγRIIb1', and FcγRIIb2, which differ only in their cytoplasmic domain sequences. All three alternatively spliced isoforms contain two extracellular Ig-like loops and a single conserved ITIM motif within their cytoplasmic tails, and are specifically included
25 within the definition of FcγRIIb, along with other splice variants that might be identified in the future.

 The terms “native FcεRI,” “native sequence FcεRI,” “native high-affinity IgE receptor FcεRI,” and “native sequence high-affinity IgE receptor FcεRI” are used interchangeably and refer to FcεRI receptors of any species, including any mammalian species, that occurs in nature.
30 FcεRI is a member of the multi-subunit immune response receptor (MIRR) family of cell surface receptors that lack intrinsic enzymatic activity but transduce intracellular signals through

association with cytoplasmic tyrosine kinases. For further details see, for example, Kinet, J.P., *Annu. Rev. Immunol.* 17:931-972 (1999) and Ott and Cambier, *J. Allergy Clin. Immunol.*, 106:429-440 (2000).

The terms "native FcεRII (CD23)," "native sequence FcεRII (CD23)," native low-affinity IgE receptor FcεRII (CD23)," "native sequence low-affinity IgE receptor FcεRII (CD23)" are used interchangeably and refer to FcεRII (CD23) receptors of any species, including any mammalian species, that occur in nature. Several groups have cloned and expressed low-affinity IgE receptors of various species. The cloning and expression of a human low-affinity IgE receptor is reported, for example, by Kikutani *et al.*, *Cell* 47:657-665 (1986), and Ludin *et al.*, *EMBO J.* 6:109-114 (1987). The cloning and expression of corresponding mouse receptors is disclosed, for example, by Gollnick *et al.*, *J. Immunol.* 144:1974-82 (1990), and Kondo *et al.*, *Int. Arch. Allergy Immunol.* 105:38-48 (1994). The molecular cloning and sequencing of CD23 for horse and cattle has been recently reported by Watson *et al.*, *Vet. Immunol. Immunopathol.* 73:323-9 (2000). For an earlier review of the low-affinity IgE receptor see also Delespesse *et al.*, *Immunol. Rev.* 125:77-97 (1992).

The term "mammal" or "mammalian species" refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, as well as rodents such as mice and rats, etc. Preferably, the mammal is human.

The term "polypeptide", in singular or plural, is used herein to refer to any peptide or protein comprising two or more amino acids joined to each other in a linear chain by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, and to longer chains, commonly referred to in the art as proteins. Polypeptides, as defined herein, may contain amino acids other than the 20 naturally occurring amino acids, and may include modified amino acids. The modification can be anywhere within the polypeptide molecule, such as, for example, at the terminal amino acids, and may be due to natural processes, such as processing and other post-translational modifications, or may result from chemical and/or enzymatic modification techniques which are well known to the art. The known modifications include, without limitation, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid

derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. Such modifications are well known to those of skill and have been described in great detail in the scientific literature, such as, for instance, Creighton, T. E., *Proteins--Structure And Molecular Properties*, 2nd Ed., W. H. Freeman and Company, New York (1993); Wold, F., "Posttranslational Protein Modifications: Perspectives and Prospects," in *Posttranslational Covalent Modification of Proteins*, Johnson, B. C., ed., Academic Press, New York (1983), pp. 1-12; Seifter et al., "Analysis for protein modifications and nonprotein cofactors," Meth. Enzymol. 182:626-646 (1990), and Rattan et al., Ann. N.Y Acad. Sci. 663:48-62 (1992).

Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. In fact, blockage of the amino or carboxyl group in a polypeptide, or both, by a covalent modification, is common in naturally occurring and synthetic polypeptides and such modifications may be present in polypeptides of the present invention, as well. For instance, the amino terminal residue of polypeptides made in *E. coli*, prior to proteolytic processing, almost invariably will be N-formylmethionine. Accordingly, when glycosylation is desired, a polypeptide is expressed in a glycosylating host, generally eukaryotic host cells. Insect cells often carry out the same post-translational glycosylations as mammalian cells and, for this reason, insect cell expression systems have been developed to express efficiently mammalian proteins having native patterns of glycosylation.

It will be appreciated that polypeptides are not always entirely linear. For instance, polypeptides may be branched as a result of ubiquitination, and they may be circular, with or without branching, generally as a result of post-translational events, including natural processing and events brought about by human manipulation which do not occur naturally. Circular, branched and branched circular polypeptides may be synthesized by non-translation natural process and by entirely synthetic methods, as well. Such structures are within the scope of the polypeptides as defined herein.

Amino acids are represented by their common one- or three-letter codes, as is common practice in the art. Accordingly, the designations of the twenty naturally occurring amino acids are as follows: Alanine=Ala (A); Arginine=Arg (R); Aspartic Acid=Asp (D); Asparagine=Asn (N); Cysteine=Cys (C); Glutamic Acid=Glu (E); Glutamine=Gln (Q); Glycine=Gly (G); Histidine=His (H); Isoleucine=Ile (I); Leucine=Leu (L); Lysine=Lys (K); Methionine=Met (M); Phenylalanine=Phe (F); Proline=Pro (P); Serine=Ser (S); Threonine=Thr (T); Tryptophan=Trp (W); Tyrosine=Tyr (Y); Valine=Val (V). The polypeptides herein may include all L-amino acids, all D-amino acids or a mixture thereof. The polypeptides comprised entirely of D-amino acids may be advantageous in that they are expected to be resistant to proteases naturally found within the human body, and may have longer half-lives.

The term "amino acid sequence variant" refers to molecules with some differences in their amino acid sequences as compared to a reference (e.g. native sequence) polypeptide. The amino acid alterations may be substitutions, insertions, deletions or any desired combinations of such changes in a native amino acid sequence.

Substitutional variants are those that have at least one amino acid residue in a native sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

Insertional variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native amino acid sequence. Immediately adjacent to an amino acid means connected to either the α -carboxy or α -amino functional group of the amino acid.

Deletional variants are those with one or more amino acids in the native amino acid sequence removed. Ordinarily, deletional variants will have at least one amino acid deleted in a particular region of the molecule.

"Sequence identity" is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in a reference polypeptide sequence (e.g., a native polypeptide sequence), after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. The % sequence identity values are generated by the NCBI BLAST2.0 software as defined by Altschul *et al.*, (1997), "Gapped BLAST and PSI-BLAST: a new

generation of protein database search programs", Nucleic Acids Res., 25:3389-3402. The parameters are set to default values, with the exception of the Penalty for mismatch, which is set to -1.

“Stringent” hybridization conditions are sequence dependent and will be different with different environmental parameters (*e.g.*, salt concentrations, and presence of organics). Generally, stringent conditions are selected to be about 5°C to 20°C lower than the thermal melting point (T_m) for the specific nucleic acid sequence at a defined ionic strength and pH. Preferably, stringent conditions are about 5°C to 10°C lower than the thermal melting point for a specific nucleic acid bound to a perfectly complementary nucleic acid. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a nucleic acid (*e.g.*, tag nucleic acid) hybridizes to a perfectly matched probe.

“Stringent” wash conditions are ordinarily determined empirically for hybridization of each set of tags to a corresponding probe array. The arrays are first hybridized (typically under stringent hybridization conditions) and then washed with buffers containing successively lower concentrations of salts, or higher concentrations of detergents, or at increasing temperatures until the signal to noise ratio for specific to non-specific hybridization is high enough to facilitate detection of specific hybridization. Stringent temperature conditions will usually include temperatures in excess of about 30° C, more usually in excess of about 37° C, and occasionally in excess of about 45° C. Stringent salt conditions will ordinarily be less than about 1000 mM, usually less than about 500 mM, more usually less than about 400 mM, typically less than about 300 mM, preferably less than about 200 mM, and more preferably less than about 150 mM. However, the combination of parameters is more important than the measure of any single parameter. *See, e.g.*, Wetmur *et al.*, J. Mol. Biol. 31:349-70 (1966), and Wetmur, Critical Reviews in Biochemistry and Molecular Biology 26(34):227-59 (1991).

In a preferred embodiment, “stringent conditions” or “high stringency conditions,” as defined herein, may be hybridization in 50% formamide, 6x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt’s solution, sonicated salmon sperm DNA (100 µg/ml), 0.5% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 2x SSC (sodium chloride/sodium citrate) and 0.1% SDS at 55°C, followed by a high-stringency wash consisting of 0.2x SSC containing 0.1% SDS at 42°C.

The term "immunoglobulin" (Ig) is used to refer to the immunity-conferring portion of the globulin proteins of serum, and to other glycoproteins, which may not occur in nature but have the same functional characteristics. The term "immunoglobulin" or "Ig" specifically includes "antibodies" (Abs). While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules that lack antigen specificity. Native immunoglobulins are secreted by differentiated B cells termed plasma cells, and immunoglobulins without any known antigen specificity are produced at low levels by the immune system and at increased levels by myelomas. As used herein, the terms "immunoglobulin," "Ig," and grammatical variants thereof are used to include antibodies, and Ig molecules without known antigen specificity, or without antigen binding regions.

Native immunoglobulins are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The main Ig isotypes (classes) found in serum, and the corresponding Ig heavy chains, shown in parentheses, are listed below:

IgG (γ chain): the principal Ig in serum, the main antibody raised in response to an antigen, has four major subtypes, several of which cross the placenta;

IgE (ϵ chain): this Ig binds tightly to mast cells and basophils, and when additionally bound to antigen, causes release of histamine and other mediators of immediate hypersensitivity; plays a primary role in allergic reactions, including hay fever, asthma and anaphylaxis; and may serve a protective role against parasites;

IgA (α chain): this Ig is present in external secretions, such as saliva, tears, mucous, and colostrum;

IgM (μ chain): the Ig first induced in response to an antigen; it has lower affinity than antibodies produced later and is pentameric; and

IgD (δ chain): this Ig is found in relatively high concentrations in umbilical cord blood, serves primarily as an early cell receptor for antigen, and is the main lymphocyte cell surface molecule.

Antibodies of the IgG, IgE, IgA, IgM, and IgD isotypes may have the same variable regions, i.e. the same antigen binding cavities, even though they differ in the constant region of their heavy chains. The constant regions of an immunoglobulin, e.g. antibody are not involved directly in binding the antibody to an antigen, but correlate with the different effector functions mediated by antibodies, such as complement activation or binding to one or more of the antibody Fc receptors expressed on basophils, mast cells, lymphocytes, monocytes and granulocytes.

Some of the main antibody isotypes (classes) are divided into further sub-classes. IgG has four known subclasses: IgG₁ (γ_1), IgG₂ (γ_2), IgG₃ (γ_3), and IgG₄ (γ_4), while IgA has two known subclasses: IgA₁ (α_1) and IgA₂ (α_2).

A light chain of an Ig molecule is either a κ or a λ chain.

The constant region of an immunoglobulin heavy chain is further divided into globular, structurally discrete domains, termed heavy chain constant domains. For example, the constant region of an IgG₁ immunoglobulin heavy chain comprises three constant domains, CH1, CH2 and CH3, and a hinge region between the CH1 and CH2 domains. The IgE immunoglobulin heavy chain comprises four constant domains: CH1, CH2, CH3 and CH4 and does not have a hinge region.

Immunoglobulin sequences, including sequences of immunoglobulin heavy chain constant regions are well known in the art and are disclosed, for example, in Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institute of Health, Bethesda, MD. (1991). For a discussion of the human IgG₁ heavy chain constant region (γ_1), see also Ellison *et al.*, *Nucl. Acid Res.* 10:4071-4079 (1982); and Takahashi *et al.*, *Cell* 29:671-679 (1982). For a discussion of the human IgG₂ constant region (γ_2), see also Krawinkel *et al.*, *EMBO J.* 1:403-407 (1982); Ellison *et al.*, *Proc. Nat. Acad. Sci. USA* 79:1984-1988 (1982); and Takahashi *et al.* (1982), *supra*. For a discussion of human IgG₃ heavy chain constant region (γ_3), see also Krawinkel *et al.*, (1982), *supra*, and Takahashi *et al.* (1982), *supra*. For a discussion of human IgG₄ heavy chain constant region (γ_4), see also Ellison *et al.*, *DNA* 1:11-18 (1982), Krawinkel *et al.* (1982), *supra*, and Takahashi *et al.* (1982), *supra*. For a discussion of the human IgE heavy chain

constant region (ϵ), see also Max *et al.*, *Cell* 29:691-699 (1982). IgE isoforms are described in Saxon *et al.*, *J. Immunol.* 147:4000 (1991); Peng *et al.*, *J. Immunol.* 148:129-136 (1992); Zhang *et al.*, *J. Exp. Med.* 176:233-243 (1992); and Hellman, *Eur. J. Immunol.* 23:159-167 (1992).

The term “allergen,” and grammatical variants thereof, are used to refer to special antigens that are capable of inducing IgE-mediated allergies. An allergen can be almost anything that acts as an antigen and stimulates an IgE-mediated allergic reaction. Common allergens can be found, for example, in food, pollen, mold, house dust which may contain mites as well as dander from house pets, venom from insects such as bees, wasps and mosquitoes.

A “Type I” allergic reaction or “immediate hypersensitivity” or “atopic allergy” occurs when an antigen entering the body encounters mast cells or basophils which have been sensitized by IgE attached to its high-affinity receptor, Fc ϵ RI on these cells. When an allergen reaches the sensitized mast cell or basophil, it cross-links surface-bound IgE, causing an increase in intracellular calcium (Ca^{2+}) that triggers the release of pre-formed mediators, such as histamine and proteases, and newly synthesized, lipid-derived mediators such as leukotrienes and prostaglandins. These autocooids produce the clinical symptoms of allergy. In addition, cytokines, e.g. IL-4, TNF-alpha, are released from degranulating basophils and mast cells, and serve to augment the inflammatory response that accompanies an IgE reaction (see, e.g. Immunology, Fifth Edition, Roitt *et al.*, eds., 1998, pp. 302-317).

The terms “vector”, “polynucleotide vector”, “construct” and “polynucleotide construct” are used interchangeably herein. A polynucleotide vector of this invention may be in any of several forms, including, but not limited to, RNA, DNA, RNA encapsulated in a retroviral coat, DNA encapsulated in an adenovirus coat, DNA packaged in another viral or viral-like form (such as herpes simplex, and adeno-associated virus (AAV)), DNA encapsulated in liposomes, DNA complexed with polylysine, complexed with synthetic polycationic molecules, conjugated with transferrin, complexed with compounds such as polyethylene glycol (PEG) to immunologically “mask” the molecule and/or increase half-life, or conjugated to a non-viral protein. Preferably, the polynucleotide is DNA. As used herein, “DNA” includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides.

1 A "host cell" includes an individual cell or cell culture which can be or has been a recipient
of any vector of this invention. Host cells include progeny of a single host cell, and the progeny
may not necessarily be completely identical (in morphology or in total DNA complement) to the
original parent cell due to natural, accidental, or deliberate mutation and/or change. A host cell
5 includes cells transfected or infected *in vivo* with a vector comprising a nucleic acid of the present
invention.

The term "promoter" means a nucleotide sequence that, when operably linked to a DNA
sequence of interest, promotes transcription of that DNA sequence.

10 Nucleic acid is "operably linked" when it is placed into a functional relationship with
another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably
linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of
the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the
transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it
15 is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA
sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in
reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by
ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide
adaptors or linkers are used in accord with conventional practice.

20 The term "IgE-mediated biological response" is used to refer to a condition or disease which
is characterized by signal transduction through an IgE receptor, including the high-affinity IgE
receptor, FcεRI, and the low-affinity IgE receptor FcεRII. The definition includes, without
limitation, conditions associated with anaphylactic hypersensitivity and atopic allergies, such as, for
example, asthma, allergic rhinitis, atopic dermatitis, food allergies, chronic urticaria and
angioedema, as well as the serious physiological condition of anaphylactic shock, usually caused by
25 bee stings or medications such as penicillin.

The terms "treat" or "treatment" refer to both therapeutic treatment and prophylactic or
preventative measures, wherein the object is to prevent or slow down (lessen) an undesired
physiological change or disorder. For purposes of this invention, beneficial or desired clinical
results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease,
30 stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression,
amelioration or palliation of the disease state, and remission (whether partial or total), whether

detectable or undetectable. Those in need of treatment include those already with the condition or disorder as well as those prone to have the condition or disorder or those in which the condition or disorder is to be prevented.

"Chronic" administration refers to administration of the agent(s) in a continuous mode as opposed to an acute mode, so as to maintain a desired effect or level of agent(s) for an extended period of time.

"Intermittent" administration is treatment that is not consecutively done without interruption, but rather is periodic in nature.

Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

An "effective amount" is an amount sufficient to effect beneficial or desired therapeutic (including preventative) results. An effective amount can be administered in one or more administrations.

"Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™, polyethylene glycol (PEG), and PLURONICS™.

II. Description of Certain Preferred Embodiments

1. Design of the fusion molecules

In one embodiment, the present invention provides fusion molecules that are capable of attenuating a biological response mediated by an FcεR, such as conditions associated with anaphylactic hypersensitivity and atopic allergies, by cross-linking an inhibitory receptor expressed on mast cells and/or basophils with an IgE receptor. The actual sequence of the fusion molecule

will depend on the targeted inhibitory receptor, such as an ITIM-containing receptor, e.g. various forms of FcγRIIb, inhibitory members of the gp49 family, especially gp49b1, p91/PIR-B, LAIR-1, LIR-1, or CD22, and on the targeted IgE receptors, e.g. FcεRI or FcεRII.

In a preferred embodiment, the inhibitory receptor is a native low-affinity FcγRIIb receptor, and the IgE receptor is a native high-affinity or low-affinity IgE receptor, i.e. FcεRI or FcεRII, more preferably FcεRI. Accordingly, the first polypeptide sequence present in the fusion molecules binds to the native low-affinity FcγRIIb receptor, while the second polypeptide sequence, which is functionally connected to the first polypeptide sequence, binds to a native FcεRI or FcεRII, preferably FcεRI. When the goal is to cross-link a native FcγRIIb receptor with a native FcεRI receptor by direct binding of the first and second polypeptide sequences present in the single-chain fusion molecules of the invention to the respective receptors, the first and second polypeptide sequences, which are functionally connected, are preferably designed to bind to the respective receptors at essentially the same region(s) as native IgG and IgE, respectively. It has been reported that the CH2-CH3 interface of the IgG Fc domain contains the binding sites for a number of Fc receptors, including the FcγRIIb low-affinity receptor (Wines *et al.*, *J. Immunol.* 164(10):5313-5318 (2000)). Based on FcεRI binding studies, Presta *et al.*, *J. Biol. Chem.* 269:26368-26373 (1994) proposed that six amino acid residues (Arg-408, Ser-411, Lys-415, Glu-452, Arg-465, and Met-469) located in three loops, C-D, E-F, and F-G, computed to form the outer ridge on the most exposed side of the human IgE heavy chain CH3 domain, are involved in binding to the high-affinity receptor FcεRI, mostly by electrostatic interactions. Helm *et al.*, *J. Cell Biol.* 271(13):7494-7500 (1996), reported that the high-affinity receptor binding site in the IgE molecule includes the Pro343-Ser353 peptide sequence within the CH3 domain of the IgE heavy chain, but sequences N- or C-terminal to this core peptide are also necessary to provide structural scaffolding for the maintenance of a receptor binding conformation. In particular, they found that residues, including His, in the C-terminal region of the ε-chain make an important contribution toward the maintenance of the high-affinity of interaction between IgE and FcεRI. The first and second polypeptide sequences within the fusion molecules of the invention are preferably designed to bind to residues within such binding regions.

In another class of the fusion molecules of the invention, the first polypeptide sequence will bind to an ITIM-containing receptor, other than FcγRIIb, expressed on mast cells, basophils and/or B cells. For example, the first polypeptide sequence may contain a region capable of specific

binding to an inhibitory member of the gp49 family, such as gp49b1, which is a member of the immunoglobulin superfamily, is preferentially expressed on mast cells and mononuclear macrophages, and contains two ITIM motifs in its cytoplasmic domain. Another ITIM-containing inhibitory receptor is p91, also referred to as PIR-B, which is known to be expressed on B cells and myeloid lineage cells. Further ITIM-containing receptors that might be targeted by the fusion molecules of the invention include, without limitation, LAIR-1, expressed on B cells, in addition to NK cells, T cells and monocytes; LIR-1, expressed on B cells and monocytes; and CD22 expressed on B cells. For review of ITIM-containing receptors and related art see, e.g. Mustelin *et al.*, Front. Biosci. 3:d1060-1096 (1998), and Sinclair *et al.*, 1999, *supra*.

When the second polypeptide sequence comprises part or whole of a native allergen protein, or a variant thereof, binding between the second polypeptide sequence and an IgE receptor occurs indirectly. The allergen-containing sequence will bind to an allergen-specific IgE molecule bound to a high-affinity IgE receptor (FcεRI) on mast cells or basophils and/or to a low-affinity IgE receptor (FcεRII, CD23) on B lymphocytes. The first, inhibitory receptor-binding, sequence is designed as discussed above. In a preferred embodiment, the allergen part of the molecule is a fragment that contains only a single IgE binding site, in order to avoid antigen cross-linking of IgE on the mast cell surface.

In a preferred embodiment, the first polypeptide sequence present in the fusion molecules of the invention has at least about 80%, more preferably at least about 85%, even more preferably at least about 90%, yet more preferably at least about 95%, most preferably at least about 99% sequence identity with the amino acid sequence of the hinge-CH₂-CH₃ region of a native IgG, e.g. IgG₁ immunoglobulin, preferably native human IgG₁. In a particularly preferred embodiment, the sequence identity is defined with reference to the human γhinge-CH₂-CH₃ sequence of SEQ ID NO: 3.

In another preferred embodiment, the first polypeptide sequence present in the fusion molecules of the invention has at least about 80%, more preferably at least about 85%, even more preferably at least about 90%, yet more preferably at least about 95%, most preferably at least about 99% sequence identity with the amino acid sequence of a native ligand of another ITIM-containing receptor expressed on mast cells, basophils and/or B cells, such as gp49b1 or p91/PIR-B (a cytoplasmic signaling protein activated by IFN-α, IFN-γ, and IL-6), or mast cell function Ag.

In yet another preferred embodiment, the first polypeptide sequence present in the fusion molecules of the invention has at least about 80%, more preferably at least about 85%, even more preferably at least about 90%, yet more preferably at least about 95%, most preferably at least about 99% sequence identity with the amino acid sequence of c-Kit (see, e.g. Yarden *et al.*, EMBO J. 6:3341-51 (1987)).

The second polypeptide sequence present in the fusion molecules of the invention preferably has at least about 80%, more preferably at least about 85%, even more preferably at least about 90%, yet more preferably at least about 95%, most preferably at least about 99% sequence identity with the amino acid sequence of the CH₂-CH₃-CH₄ region of a native IgE immunoglobulin, preferably native human IgE, or with the sequence of a native allergen protein. In a particularly preferred embodiment, the sequence identity is defined with reference to the human CH₂-CH₃-CH₄ sequence of SEQ ID NO: 6 or with regard to one of the allergen sequences listed in Table 1 below (SEQ ID NOS: 7 through 173), or, in a preferred embodiment, one of two Ara h2 clones represented by SEQ ID NOS: 174 and 175, respectively.

Alternatively, the first polypeptide sequence present in the fusion molecules of the invention may comprise a sequence encoded by nucleic acid hybridizing under stringent conditions to the complement of the coding sequence of a native γ hinge-CH₂-CH₃ sequence, preferably the γ hinge-CH₂-CH₃ coding sequence from within SEQ ID NO: 1, or with the coding sequence of another immunoglobulin heavy chain constant region sequence required for IgG binding.

When the first polypeptide sequence binds specifically to an ITIM-containing receptor expressed on mast cells, basophils or B cells, it is preferably encoded by nucleic acid hybridizing under stringent conditions to the complement of the coding sequence of a native ligand of that receptor.

Similarly, the second polypeptide sequence present in the fusion molecules of the invention may comprise a sequence encoded by nucleic acid hybridizing under stringent conditions to the complement of the coding sequence of a native CH₂-CH₃-CH₄ sequence, preferably the CH₂-CH₃-CH₄ coding sequence from within SEQ ID NO: 4, or to the complement of the coding sequence of a native allergen, such as those listed in Table 1.

Whenever the first and/or second polypeptide sequence included in the fusion molecules of the invention is an amino acid variant of a native immunoglobulin constant region sequence, it is required to retain the ability to bind to the corresponding native receptor, such as a native IgG

inhibitory receptor (e.g. FcγRIIb) and a native high-affinity IgE receptor (e.g. FcεRI) or native low-affinity IgE receptor (FcεRII, CD23), respectively. As discussed above, the receptor binding domains within the native IgG and IgE heavy chain constant region sequences have been identified. Based on this knowledge, the amino acid sequence variants may be designed to retain the native amino acid residues essential for receptor binding, or to perform only conservative amino acid alterations (e.g. substitutions) at such residues.

In making amino acid sequence variants that retain the required binding properties of the corresponding native sequences, the hydropathic index of amino acids may be considered. For example, it is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score without significant change in biological activity. Thus, isoleucine, which has a hydropathic index of + 4.5, can generally be substituted for valine (+ 4.2) or leucine (+ 3.8), without significant impact on the biological activity of the polypeptide in which the substitution is made. Similarly, usually lysine (-3.9) can be substituted for arginine (-4.5), without the expectation of any significant change in the biological properties of the underlying polypeptide.

Other considerations for choosing amino acid substitutions include the similarity of the side-chain substituents, for example, size, electrophilic character, charge in various amino acids. In general, alanine, glycine and serine; arginine and lysine; glutamate and aspartate; serine and threonine; and valine, leucine and isoleucine are interchangeable, without the expectation of any significant change in biological properties. Such substitutions are generally referred to as conservative amino acid substitutions, and, as noted above, are the preferred type of substitutions within the polypeptides of the present invention.

Alternatively or in addition, the amino acid alterations may serve to enhance the receptor binding properties of the fusion molecules of the invention. Variants with improved receptor binding and, as a result, superior biological properties can be readily designed using standard mutagenesis techniques, such as alanine-scanning mutagenesis, PCR mutagenesis or other mutagenesis techniques, coupled with receptor binding assays, such as the assay discussed below or described in the Example.

In a preferred embodiment, the fusion molecules of the present invention comprise a first polypeptide sequence including functionally active hinge, CH2 and CH3 domains of the constant region of an IgG₁ heavy chain (γhinge-CH₂-CH₃ sequence) linked at its C-terminus to the N-terminus of a second polypeptide including functionally active CH2, CH3 and CH4 domains of the

constant region of an IgE heavy chain (CH ϵ 2-CH ϵ 3-CH ϵ 4 sequence). In a particularly preferred embodiment, the first polypeptide sequence is composed of functionally active hinge, CH2 and CH3 regions of a native human IgG $_1$ heavy chain, linked at its C-terminus to the N-terminus of a second polypeptide composed of functionally active CH2, CH3 and CH4 domains of a native human IgE heavy chain constant region.

While it is preferred to fuse the IgG heavy chain constant region sequence (or a homologous sequence) C-terminally to the N-terminus of the IgE heavy chain constant region sequence (or a homologous sequence), fusion molecules in which the IgE heavy chain constant region sequence (or a homologous sequence) is fused C-terminally to the N-terminus of the IgG heavy chain constant region sequence (or a homologous sequence) are also within the scope of the invention. The fusion molecules may also comprise repeats of identical or different IgG and/or IgE heavy chain constant region sequences. For example, two repeats of IgG heavy chain constant region sequences, each including an IgG inhibitory receptor-binding domain, can be followed by IgE heavy chain constant region sequences (GGE structure), or two repeats of identical or different IgG heavy chain constant region sequences may flank an IgE heavy chain constant region sequence (GEG structure), etc. Fusion molecules comprising more than one binding sequence for a target receptor (e.g. an Fc γ RIIb receptor) are expected to have superior biological, e.g. anti-allergic properties.

The same considerations apply to the structure of fusion molecules where the second polypeptide sequence comprises, is or is derived from an allergen protein. Such molecules may also include repeats of the IgG heavy chain constant region sequences, fused to either or both sides of the allergen sequence.

Similarly, molecules in which the first polypeptide sequence binds to a different inhibitory receptor expressed on mast cells and/or basophils, e.g. an ITIM-containing inhibitory receptor functionally connected to a second polypeptide sequence binding directly or indirectly to an IgE receptor, e.g. Fc ϵ RI, may contain multiple repeats of the inhibitory receptor binding regions and/or the IgE binding regions.

In all embodiments, the two polypeptide sequences are functionally connected, which means that they retain the ability to bind to the respective native receptors, such as a native IgG inhibitory receptor, e.g. a low-affinity Fc γ RIIb receptor, and to a native high-affinity IgE receptor, e.g. Fc ϵ RI or low-affinity IgE receptor, as desired. As a result, the fusion molecules, comprising the first and second polypeptide sequences functionally connected to each other, are capable of cross-

linking the respective native receptors, such as FcγRIIb and FcεRI or FcγRIIb and FcεRII. In order to achieve a functional connection between the two binding sequences within the fusion molecules of the invention, it is preferred that they retain the ability to bind to the corresponding receptor with a binding affinity similar to that of a native immunoglobulin ligand of that receptor.

5 The fusion molecules of the present invention are typically produced and act as homodimers or heterodimers, comprising two of the fusion molecules hereinabove described covalently linked to each other. The covalent attachment is preferably achieved via one or more disulfide bonds. For example, the prototype protein designated GE2 is produced as a homodimer composed of the two γhinge-CHγ2-CHγ3-15aa linker-CHε2-CHε3-CHε4 chains connected to each other by interchain disulfide bonds, to provide an immunoglobulin-like structure. It is also possible to produce heterodimers, in which two different fusion molecules are linked to each other by one or more covalent linkages, e.g. disulfide bond(s). Such bifunctional structures might be advantageous in that they are able to cross-link the same or different IgεR(s) with different inhibitory receptors.

15 Receptor binding can be tested using any known assay method, such as competitive binding assays, direct and indirect sandwich assays. Thus, binding of a first polypeptide sequence included in the fusion molecules herein to a low-affinity IgG inhibitory receptor, or the binding of a second polypeptide sequence included herein to a high-affinity or low-affinity IgE receptor can be tested using conventional binding assays, such as competitive binding assays, including RIAs and ELISAs. Ligand/receptor complexes can be identified using traditional separation methods as filtration, centrifugation, flow cytometry, and the results from the binding assays can be analyzed using any conventional graphical representation of the binding data, such as Scatchard analysis. The assays may be performed, for example, using a purified receptor, or intact cells expressing the receptor. One or both of the binding partners may be immobilized and/or labeled. A particular cell-based binding assay is described in the Example below.

25 The two polypeptide sequences present in the fusion molecules of the invention may be associated with one another by any means that allows them to cross-link the relevant receptors. Thus, association may take place by a direct or indirect covalent linkage, where "indirect" covalent linkage means that the two polypeptide sequences are part of separate molecules that interact with one another, either directly or indirectly. For example, each polypeptide sequence can be directly linked to one member of an interacting pair of molecules, such as, for example, a biotin/avidin pair.

Alternatively, the two polypeptide sequences can be linked using a "dimerizer" system based on linkage to an entity that associates with a common ligand, such as dimerizer systems based on cyclosporin, FK506, rapamycin, countermycin, and the like.

In a preferred embodiment, the first and second polypeptide sequences, such as, for example, two immunoglobulin constant region segments, or an immunoglobulin constant region sequence and an allergen sequence, are connected by a polypeptide linker. The polypeptide linker functions as a "spacer" whose function is to separate the functional receptor binding domains, or the Fcγ receptor binding domain and the IgE-binding sequence in the allergen, so that they can independently assume their proper tertiary conformation. The polypeptide linker usually comprises between about 5 and about 25 residues, and preferably contains at least about 10, more preferably at least about 15 amino acids, and is composed of amino acid residues which together provide a hydrophilic, relatively unstructured region. Linking amino acid sequences with little or no secondary structure work well. The specific amino acids in the spacer can vary, however, cysteines should be avoided. Suitable polypeptide linkers are, for example, disclosed in W0 88/09344 (published on December 1, 1988), as are methods for the production of multifunctional proteins comprising such linkers.

In a less preferred embodiment, the IgG and IgE constant region sequences, the IgG constant region sequences and the allergen sequences, or sequences showing high degree of sequence identity with such sequences, may be directly fused to each other, or connected by non-polypeptide linkers. Such linkers may, for example, be residues of covalent bifunctional cross-linking agents capable of linking the two sequences without the impairment of the receptor (antibody) binding function. The bifunctional cross-linking reagents can be divided according to the specificity of their functional groups, e.g. amino, sulfhydryl, guanidino, indole, carboxyl specific groups. Of these, reagents directed to free amino groups have become especially popular because of their commercial availability, ease of synthesis and the mild reaction conditions under which they can be applied. A majority of heterobifunctional cross-linking reagents contains a primary amine-reactive group and a thiol-reactive group (for review, see Ji, T. H. "Bifunctional Reagents" in: *Meth. Enzymol.* 91:580-609 (1983)).

In a further specific embodiment, the two polypeptide sequences (including variants of the native sequences) are dimerized by amphiphilic helices. It is known that recurring copies of the amino acid leucine (Leu) in gene regulatory proteins can serve as teeth that "zip" two protein molecules together to provide a dimer. For further details about leucine zippers, which can serve as

linkers for the purpose of the present invention, see for example: Landschulz, W. H., *et al. Science* 240:1759-1764 (1988); O'Shea, E. K. *et al., Science* 243: 38-542 (1989); McKnight, S. L., *Scientific American* 54-64, April 1991; Schmidt-Dorr, T. *et al., Biochemistry* 30:9657-9664 (1991); Blondel, A. and Bedouelle, H. *Protein Engineering* 4:457-461 (1991), and the references cited in these papers.

In a different approach, the two polypeptide sequences (including variants of the native sequences) are linked via carbohydrate-directed bifunctional cross-linking agents, such as those disclosed in U.S. Patent No. 5,329,028.

The cross-linking of an inhibitory receptor expressed on mast cells and/or basophils, such as an ITIM-containing receptor, including IgG inhibitory receptors, e.g. FcγRIIb and a high-affinity IgE receptor, e.g. FcεRI or low-affinity IgE receptor, e.g. FcεRII, inhibit FcεR mediated biological responses. Such biological responses preferably are the mediation of an allergic reactions or autoimmune reactions via FcεR, including, without limitation, conditions associated with IgE mediated reactions, such as, for example, asthma, allergic rhinitis, food allergies, chronic urticaria and angioedema, allergic reactions to hymenophthera (e.g. bee and yellow jacket) stings or medications such as penicillin up to and including the severe physiological reaction of anaphylactic shock.

2. Preparation of the fusion molecules

When the fusion molecules are polypeptides, in which the first and second polypeptide sequences are directly fused or functionally connected by a polypeptide linker, they can be prepared by well known methods of recombinant DNA technology or traditional chemical synthesis. If the polypeptides are produced by recombinant host cells, cDNA encoding the desired polypeptide of the present invention is inserted into a replicable vector for cloning and expression. As discussed before, the nucleotide and amino acid sequences of native immunoglobulin constant regions, including native IgG and IgE constant region sequences, are well known in the art and are readily available, for example, from Kabat *et al., Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institute of Health, Bethesda, MD. (1991).

Similarly, the sequences of a large number of allergens are well known in the art. According to a nomenclature system established for allergens by the WHO/IUIS Allergen Nomenclature Subcommittee, the designation of any particular allergen is composed of the first three letters of the genus; a space; the first letter of the species name; a space and an arabic number.

In the event that two species names have identical designations, they are discriminated from one another by adding one or more letters to each species designation. Using this designation, the allergen Aln G 1 is a major pollen allergen from the genus Alnus and the species glutinosa, the sequence of which is available from the SWISS-PROT database under the entry name MPAC_ALNGL (Primary Accession number: P38948) (Breitender *et al.*, *J. Allergy Clin. Immunol.* 90:909-917 (1992)). A list of known antigens, including their origin, entry name and Primary Accession Number in the SWISS-PROT database is provided in Table 1. The molecular weight of most food allergens is between 10,000 and 70,000 Da. Some allergens, such as Ara h 1 (63.5 kDa) and Ara h 2 (17kDa), occur as polymers that are larger, e.g. 200 to 300 kDa.

As noted earlier, it might be advantageous to use in the fusion molecules of the present invention a fragment of a native or variant allergen that contains only a single IgE-binding site. For many of the allergen proteins listed in Table 1, the IgE-binding sites have been determined. For example, the IgE-binding epitopes of Par j 2, a major allergen of *Parietaria judaica* pollen, have been determined by Costa *et al.*, *Allergy* 55:246-50 (2000). The IgE-binding epitopes of major peanut antigens Ara h 1 (Burks *et al.*, *Eur. J. Biochem.* 254:334-9 (1997)); Ara h 2 (Stanley *et al.*, *Arch Biochem. Biophys.* 342:244-53 (1997)); and Ara h 3 (Rabjohn *et al.*, *J. Clin. Invest.* 103:535-42 (1999)) are also known, just to mention a few.

TABLE 1

Allergen	SWISS-PROT Entry	SWISS-PROT Accession No.	Protein Name	Source	SEQ ID NO.
Aln g 1	MPAG_ALNGL	P38948	Major Pollen Allergen Aln g 1	Pollen of <i>Alnus glutinosa</i> (Alder)	8
Alt a 6	RLA2_ALTAL	P42037	60S Acidic Ribosomal Protein P2	<i>Alternaria alternata</i>	9
Alt a 7	ALA7_ALTAL	P42058	Minor Allergen Alt a 7	<i>Alternaria alternata</i>	10
Alt a 10	DHAL_ALTAL	P42041	Aldehyde Dehydrogenase	<i>Alternaria alternata</i>	11
Alt a 12	RLA1_ALTAL	P49148	60S Acidic Ribosomal Protein P1	<i>Alternaria alternata</i>	12
Amb a 1	MP11_AMBAR	P27759	Pollen Allergen Amb a 1.1 [Precursor]	<i>Ambrosia artemisiifolia</i> (Short ragweed)	13
Amb a 1	MP12_AMBAR	P27760	Pollen Allergen Amb a 1.2 [Precursor]	<i>Ambrosia artemisiifolia</i> (Short ragweed)	14
Amb a 1	MP13_AMBAR	P27761	Pollen Allergen Amb a 1.3 [Precursor]	<i>Ambrosia artemisiifolia</i> (Short ragweed)	15
Amb a 1	MP14_AMBAR	P28744	Pollen Allergen Amb a 1.4 [Precursor]	<i>Ambrosia artemisiifolia</i> (Short ragweed)	16
Amb a 2	MPA2_AMBAR	P27762	Pollen Allergen Amb a 2 [Precursor]	<i>Ambrosia artemisiifolia</i> (Short ragweed)	17
Amb a 3	MPA3_AMBEL	P00304	Pollen Allergen Amb a 3	<i>Ambrosia artemisiifolia</i> var. <i>elatior</i> (Short ragweed)	18
Amb a 5	MPA5_AMBEL	P02878	Pollen Allergen Amb a 5	<i>Ambrosia artemisiifolia</i> var. <i>elatior</i> (Short ragweed)	19
Amb p 5	MPA5_AMBPS	P43174	Pollen Allergen Amb p 5-a [Precursor]	<i>Ambrosia psilostachya</i> (Western ragweed)	20
Amb p 5	MP5B_AMBPS	P43175	Pollen Allergen Amb p 5b [Precursor]	<i>Ambrosia psilostachya</i> (Western ragweed)	21

Amb t 5	MPT5_AMBTR	P10414	Pollen Allergen Amb t 5 [Precursor]	Ambrosia trifida (Giant ragweed)	22
Api g 1	MPAG_APIGR	P49372	Major Allergen Api g 1	Apium graveolens (Celery)	23
Api m 1	PA2_APIME	P00630	Phospholipase A2 [Precursor] [Fragment]	Apis mellifera (Honeybee)	24
Api m 2	HUGA_APIME	Q08169	Hyaluronoglucosaminidase [Precursor]	Apis mellifera (Honeybee)	25
Api m 3	MEL_APIME	P01501	Melittin [Precursor]	Apis mellifera (Honeybee) Apis cerana (Indian honeybee)	26
Ara h 1	AH11_ARAHY	P43237	Allergen Ara h 1, Clone P17	Arachis hypogaea (Peanut)	27
Ara h 1	AH12_ARAHY	P43238	Allergen Ara h 1, Clone P41b	Arachis hypogaea (Peanut)	28
Ara t 8	PRO1_ARATH	Q42449	Profilin 1	Arabidopsis thaliana (Mouse-ear cress)	29
Asp f 1	RNMG_AS PRE	P04389	Ribonuclease Mitogillin [Precursor]	Aspergillus restrictus; Aspergillus fumigatus (Sartorya fumigata)	30
Asp f 2	MAF2_AS PFU	P79017	Major Allergen Asp f 2 [Precursor]	Aspergillus fumigatus (Sartorya fumigata)	31
Asp f 3	PM20_AS PFU	O43099	Probable Peroxisomal Membrane Protein PMP20	Aspergillus fumigatus (Sartorya fumigata)	32
Asp f 13	AF13_AS PFU	O60022	Allergen Asp f 13 [Precursor]	Aspergillus fumigatus (Sartorya fumigata)	33
Bet v 1	BV1A_BETVE	P15494	Major Pollen Allergen Bet v 1-a	Betula verrucosa (White birch) (Betula pendula)	34
Bet v 1	BV1C_BETVE	P43176	Major Pollen Allergen Bet v 1-c	Betula verrucosa (White birch) (Betula pendula)	35
Bet v 1	BV1D_BETVE	P43177	Major Pollen Allergen Bet v 1-d/h	Betula verrucosa (White birch) (Betula pendula)	36
Bet v 1	BV1E_BETVE	P43178	Major Pollen Allergen Bet v 1-e	Betula verrucosa (White birch) (Betula pendula)	37
Bet v 1	BV1F_BETVE	P43179	Major Pollen	Betula verrucosa	38

			Allergen Bet v 1-f/i	(White birch) (Betula pendula)	
Bet v 1	BV1G_BETVE	P43180	Major Pollen Allergen Bet v 1-g	Betula verrucosa (White birch) (Betula pendula)	39
Bet v 1	BV1J_BETVE	P43183	Major Pollen Allergen Bet v 1-j	Betula verrucosa (White birch) (Betula pendula)	40
Bet v 1	BV1K_BETVE	P43184	Major Pollen Allergen Bet v 1-k	Betula verrucosa (White birch) (Betula pendula)	41
Bet v 1	BV1L_BETVE	P43185	Major Pollen Allergen Bet v 1-l	Betula verrucosa (White birch) (Betula pendula)	42
Bet v 1	BV1M_BETVE	P43186	Major Pollen Allergen Bet v 1-m/n	Betula verrucosa (White birch) (Betula pendula)	43
Bet v 2	PROF-BETVE	P25816	Profilin	Betula verrucosa (White birch) (Betula pendula)	44
Bet v 3	BTV3_BETVE	P43187	Allergen Bet v 3	Betula verrucosa (White birch) (Betula pendula)	45
Bla g 2	ASP2_BLAG	P54958	Aspartic Protease Bla g 2 [Precursor]	Blattella germanica (German cockroach)	46
Bla g 4	BLG4_BLAG	P54962	Allergen Bla g 4 [Precursor] [Fragment]	Blattella germanica (German cockroach)	47
Bla g 5	GTS1_BLAG	O18598	Glutathione-S-transferase	Blattella germanica (German cockroach)	48
Blo t 12	BT12_BLOTA	Q17282	Allergen Blo t 12 [Precursor]	Blomia tropicalis (Mite)	49
Bos d 2	ALL2_BOVIN	Q28133	Allergen Bos d 2 [Precursor]	Bos taurus (Bovine)	50
Bos d 5	LACB_BOVIN	P02754	Beta-lactoglobulin [Precursor]	Bos taurus (Bovine)	51
Bra j 1	ALL1_BRAJU	P80207	Allergen Bra j 1-e, Small and Large Chains	Brassica juncea (Leaf mustard) (Indian mustard)	52
Can a 1	ADH1_CANAL	P43067	Alcohol Dehydrogenase 1	Candida albicans (Yeast)	53
Can f 1	ALL1_CANFA	O18873	Major Allergen Can f 1 [Precursor]	Canis familiaris (Dog)	54

Can f 2	ALL2_CANFA	O18874	Minor Allergen Can f 2 [Precursor]	Canis familiaris (Dog)	55
Car b 1	MPA1_CARBE	P38949	Major Pollen Allergen Car b 1, Isoforms 1A and 1B	Carpinus betulus (Hornbeam)	56
Car b 1	MPA2_CARBE	P38950	Major Pollen Allergen Car b 1, Isoform 2	Carpinus betulus (Hornbeam)	57
Cha o 1	MPA1_CHAOB	Q96385	Major Pollen Allergen Cha o 1 [Precursor]	Chamaecyparis obtusa (Japanese cypress)	58
Cla h 3	DHAL_CLAHE	P40108	Aldehyde Dehydrogenase	Cladosporium herbarum	59
Cla h 3	RLA3_CLAHE	P42038	60S Acidic Ribosomal Protein P2	Cladosporium herbarum	60
Cla h 4	HS70_CLAHE	P40918	Heat Shock 70 KDa Protein	Cladosporium herbarum	61
Cla h 4	RLA4_CLAHE	P42039	60S Acidic Ribosomal Protein P2	Cladosporium herbarum	62
Cla h 5	CLH5_CLAHE	P42059	Minor Allergen Cla h 5	Cladosporium herbarum	63
Cla h 6	ENO_CLAHE	P42040	Enolase	Cladosporium herbarum	64
Cla h 12	RLA1_CLAHE	P50344	60S Acidic Ribosomal Protein P1	Cladosporium herbarum	65
Cop c 2	THIO_CAPCM				
Cor a 1	MPAA_CORAV	Q08407	Major Pollen Allergen Cor a 1, Isoforms 5, 6, 11 and 16	Corylus avellana (European hazel)	66
Cup a 1	MPA1_CUPAR	Q9SCG9	Major Pollen Allergen Cup a 1	Cupressus arizonica	67
Cry j 1	SBP_CRYJA	P18632	Sugi Basic Protein [Precursor]	Cryptomeria japonica (Japanese cedar)	68
Cry j 2	MPA2_CRYJA	P43212	Possible Polygalacturonase	Cryptomeria japonica (Japanese cedar)	69
Cyn d 12	PROF_CYNDA	O04725	Profilin	Cynodon dactylon (Bermuda grass)	70
Dac g 2	MPG2_DACGL	Q41183	Pollen Allergen Dac g 2 [Fragment]	Dactylis glomerata (Orchard grass) (Cocksfoot grass)	71
Dau c 1	DAU1_DAUCA	O04298	Major Allergen Dau c	Daucus carota	72

			1	(Carrot)	
Der f 1	MMAL_DERFA	P16311	Major Mite Fecal Allergen Der f 1 [Precursor]	Dermatophagoides farinae (House-dust mite)	73
Der f 2	DEF2_DERFA	Q00855	Mite Allergen Der f 2 [Precursor]	Dermatophagoides ferinae (House-dust mite)	74
Der f 3	DEF3_DERFA	P49275	Mite Allergen Der f 3 [Precursor]	Dermatophagoides ferinae (House-dust mite)	75
Der f 6	DEF6_DERFA	P49276	Mite Allergen Der f 6 [Fragment]	Dermatophagoides ferinae (House-dust mite)	76
Der f 7	DEF7_DERFA	Q26456	Mite Allergen Der f 7 [Precursor]	Dermatophagoides ferinae (House-dust mite)	77
Der m 1	MMAL_DERMI	P16312	Major Mite Fecal Allergen Der m 1 [Fragment]	Dermatophagoides microceras (House-dust mite)	78
Der p 1	MMAL_DERPT	P08176	Major Mite Fecal Allergen Der p 1 [Precursor]	Dermatophagoides pteronyssinus (House-dust mite)	79
Der p 2	DER2_DERPT	P49278	Mite Allergen Der p 2 [Precursor]	Dermatophagoides pteronyssinus (House-dust mite)	80
Der p 3	DER3_DERPT	P39675	Mite Allergen Der p 3 [Precursor]	Dermatophagoides pteronyssinus (House-dust mite)	81
Der p 4	AMY_DERPT	P49274	Alpha-Amylase [Fragment]	Dermatophagoides pteronyssinus (House-dust mite)	82
Der p 5	DER5_DERPT	P14004	Mite Allergen Der p 5	Dermatophagoides pteronyssinus (House-dust mite)	83
Der p 6	DER6_DERPT	P49277	Mite Allergen Der p 6 [Fragment]	Dermatophagoides pteronyssinus (House-dust mite)	84
Der p 7	DER7_DERPT	P49273	Mite Allergen Der p 7 [Precursor]	Dermatophagoides pteronyssinus (House-dust mite)	85
Dol a 5	VA5_DOLAR	Q05108	Venom Allergen 5	Dolichovespula arenaria (Yellow hornet)	86
Dol m 1	PA11_DOLMA	Q06478	Phospholipase A1 1 [Precursor] [Fragment]	Dolichovespula maculata (White-face hornet) (Bald-faced	87

				hornet)	
Dol m 1	PA12_DOLMA	P53357	Phospholipase A1 2	Dolichovespula maculata (White-face hornet) (Bald-faced hornet)	88
Dol m 2	HUGA_DOLMA	P49371	Hyaluronoglucosaminidase	Dolichovespula maculata (White-face hornet) (Bald-faced hornet)	89
Dol m 5	VA52_DOLMA	P10736	Venom Allergen 5.01 [Precursor]	Dolichovespula maculata (White-face hornet) (Bald-faced hornet)	90
Dol m 5	VA53_DOLMA	P10737	Venom Allergen 5.02 [Precursor] [Fragment]	Dolichovespula maculata (White-face hornet) (Bald-faced hornet)	91
Equ c 1	ALL1_HORSE	Q95182	Major Allergen Equ c 1 [Precursor]	Equus caballus (Horse)	92
Equ c 2	AL21_HORSE	P81216	Dander major Allergen Equ c 2.0101 [Fragment]	Equus caballus (Horse)	93
Equ c 2	AL22_HORSE	P81217	Dander Major Allergen Equ c 2.0102 [Fragment]	Equus caballus (Horse)	94
Eur m 1	EUM1_EURMA	P25780	Mite Group I Allergen Eur m 1 [Fragment]	Euroglyphus maynei (House-dust mite)	95
Fel d 1	FELA_FELCA	P30438	Major Allergen I Polypeptide Chain 1 Major Form [Precursor]	Felis silvestris catus (Cat)	96
Fel d 1	FELB_FELCA	P30439	Major Allergen I Polypeptide Chain 1 Minor Form [Precursor]	Felis silvestris catus (Cat)	97
Fel d 1	FEL2_FELCA	P30440	Major Allergen I Polypeptide Chain 2 [Precursor]	Felis silvestris catus (Cat)	98
Gad c 1	PRVB_GADCA	P02622	Parvalbumin Beta	Gadus callarias (Baltic cod)	99
Gal d 1	IOVO_CHICK	P01005	Ovomucoid [Precursor]	Gallus gallus (Chicken)	100
Gal d 2	OVAL_CHICK	P01012	Ovalbumin	Gallus gallus (Chicken)	101

Gal d 3	TRFE_CHICK	P02789	Ovotransferrin [Precursor]	Gallus gallus (Chicken)	102
Gal d 4	LYC_CHICK	P00698	Lysozyme C [Precursor]	Gallus gallus (Chicken)	103
Hel a 2	PROF_HELAN	O81982	Profilin	Helianthus annuus (Common sunflower)	104
Hev b 1	REF_HEVBR	P15252	Rubber Elongation Factor Protein	Hevea brasiliensis (Para rubber tree)	105
Hev b 5	HEV5_HEVBR	Q39967	Major Latex Allergen Hev b 5	Hevea brasiliensis (Para rubber tree)	106
Hol l 1	MPH1_HOLLA	P43216	Major Pollen Allergen Hol l 1 [Precursor]	Holcul lanatus (Velvet grass)	107
Hor v 1	IAA1_HORVU	P16968	Alpha-amylase Inhibitor Bmai-1 [Precursor] [Fragment]	Hordeum vulgare (Barley)	108
Jun a 1	MPA1_JUNAS	P81294	Major Pollen Allergen Jun a 1 [Precursor]	Juniperus ashei (Ozark white cedar)	109
Jun a 3	PRR3_JUNAS	P81295	Pathogenesis-Related Protein [Precursor]	Juniperus ashei (Ozark white cedar)	110
Lep d 1	LEP1_LEPDS	P80384	Mite Allergen Lep d 1 [Precursor]	Lepidoglyphus destructor (Storage mite)	111
Lol p 1	MPL1_LOLPR	P14946	Pollen Allergen Lol p 1 [Precursor]	Lolium perenne (Perennial ryegrass)	112
Lol p 2	MPL2_LOLPR	P14947	Pollen Allergen Lol p 2-a	Lolium perenne (Perennial ryegrass)	113
Lol p 3	MPL3_LOLPR	P14948	Pollen Allergen Lol p 3	Lolium perenne (Perennial ryegrass)	114
Lol p 5	MP5A_LOLPR	Q40240	Major Pollen Allergen Lol p 5a [Precursor]	Lolium perenne (Perennial ryegrass)	115
Lol p 5	MP5B_LOLPR	Q40237	Major Pollen Allergen Lol p 5b [Precursor]	Lolium perenne (Perennial ryegrass)	116

Mal d 1	MAL1_MALDO	P43211	Major Allergen Mal d 1	Malus domestica (Apple) (Malus sylvestris)	117
Mer a 1	PROF_MERAN	O49894	Profilin	Mercurialis annua (Annual mercury)	118
Met e 1	TPM1_METEN	Q25456	Tropomyosin	Metapenaeus ensis (Greasyback shrimp) (Sand shrimp)	119
Mus m 1	MUP6_MOUSE	P02762	Major Urinary Protein 6 [Precursor]	Mus musculus (Mouse)	120
Myr p 1	MYR1_MYRPI	Q07932	Major Allergen Myr p 1 [Precursor]	Myrmecia pilosula (Bulldog ant) (Australian jumper ant)	121
Myr p 2	MYR2_MYRPI	Q26464	Allergen Myr p 2 [Precursor]	Myrmecia pilosula (Bulldog ant) (Australian jumper ant)	122
Ole e 1	ALL1_OLEEU	P19963	Major Pollen Allergen	Olea europaea (Common olive)	123
Ole e 4	ALL4_OLEEU	P80741	Major Pollen Allergen Ole e 4 [Fragments]	Olea europaea (Common olive)	124
Ole e 5	SODC_OLEEU	P80740	Superoxide Dismutase [CU-ZN] [Fragment]	Olea europaea (Common olive)	125
Ole e 7	ALL7_OLEEU	P81430	Pollen Allergen Ole e 7 [Fragment]	Olea europaea (Common olive)	126
Ory s 1	MPO1_ORYSA	Q40638	Major Pollen Allergen Ory s 1 [Precursor]	Oryza sativa (Rice)	127
Par j 1	NL11_PARJU	P43217	Probable Nonspecific Lipid-Transfer Protein [Fragment]	Parietaria judaica	128
Par j 1	NL12_PARJU	O04404	Probable Nonspecific Lipid-Transfer Protein 1 [Precursor]	Parietaria judaica	129
Par j 1	NL13_PARJU	Q40905	Probable Nonspecific Lipid-Transfer Protein 1 [Precursor]	Parietaria judaica	130
Par j 2	NL21_PARJU	P55958	Probable Nonspecific Lipid-Transfer	Parietaria judaica	131

			Protein 2 [Precursor]		
Par j 2	NL22_PARJU	O04403	Probable Nonspecific Lipid-Transfer Protein 2 [Precursor]	Parietaria judaica	132
Pha a 1	MPA1_PHAAQ	Q41260	Major Pollen Allergen Pha a 1 [Precursor]	Phalaris aquatica (Canary grass)	133
Pha a 5	MP51_PHAAQ	P56164	Major Pollen Allergen Pha a 5.1 [Precursor]	Phalaris aquatica (Canary grass)	134
Pha a 5	MP52_PHAAQ	P56165	Major Pollen Allergen Pha a 5.2 [Precursor]	Phalaris aquatica (Canary grass)	135
Pha a 5	MP53_PHAAQ	P56166	Major Pollen Allergen Pha a 5.3 [Precursor]	Phalaris aquatica (Canary grass)	136
Pha a 5	MP54_PHAAQ	P56167	Major Pollen Allergen Pha a 5.4 [Fragment]	Phalaris aquatica (Canary grass)	137
Phl p 1	MPP1_PHLPR	P43213	Pollen Allergen Phl p 1 [Precursor]	Phleum pratense (Common timothy)	138
Phl p 2	MPP2_PHLPR	P43214	Pollen Allergen Phl p 2 [Precursor]	Phleum pratense (Common timothy)	139
Phl p 5	MP5A_PHLPR	Q40962	Pollen Allergen Phl p 5a [Fragment]	Phleum pratense (Common timothy)	140
Phl p 5	MP5B_PHLPR	Q40963	Pollen Allergen Phl p 5b [Precursor] [Fragment]	Phleum pratense (Common timothy)	141
Phl p 6	MPP6_PHLPR	P43215	Pollen Allergen Phl p 6 [Precursor]	Phleum pratense (Common timothy)	1412
Phl p 11	PRO1_PHLPR	P35079	Profilin 1	Phleum pratense (Common timothy)	143
Phl p 11	PRO2_PHLPR	O24650	Profilin 2/4	Phleum pratense (Common timothy)	144
Phl p 11	PRO3_PHLPR	O24282	Profilin 3	Phleum pratense (Common timothy)	145
Poa p 9	MP91_POAPR	P22284	Pollen Allergen Kbg	Poa pratensis	146

			31 [Precursor]	(Kentucky bluegrass)	
Poa p 9	MP92_POAPR	P22285	Pollen Allergen Kbg 41 [Precursor]	Poa pratensis (Kentucky bluegrass)	147
Poa p 9	MP93_POAPR	P22286	Pollen Allergen Kbg 60 [Precursor]	Poa pratensis (Kentucky bluegrass)	148
Pol a 5	VA5_POLAN	Q05109	Venom Allergen 5 [Precursor] [Fragment]	Polistes annularis (Paper wasp)	149
Pol d 5	VA5_POLDO	P81656	Venom Allergen 5	Polistes dominulus (European paper wasp)	150
Pol e 5	VA5_POLEX	P35759	Venom Allergen 5	Polistes exclamans (Paper wasp)	151
Pol f 5	VA5_POLFU	P35780	Venom Allergen 5	Polistes fuscatus (Paper wasp)	152
Pru a 1	PRU1_PRUAV	O24248	Major Allergen Pru a 1	Prunus avium (Cherry)	153
Rat n 1	MUP_RAT	P02761	Major Urinary Protein [Precursor]	Rattus norvegicus (Rat)	154
Sol i 2	VA2_SOLIN	P35775	Venom Allergen II [Precursor]	Solenopsis invicta (Red imported fire ant)	155
Sol i 3	VA3_SOLIN	P35778	Venom Allergen III	Solenopsis invicta (Red imported fire ant)	156
Sol i 4	VA4_SOLIN	P35777	Venom Allergen IV	Solenopsis invicta (Red imported fire ant)	157
Sol r 2	VA2_SOLRI	P35776	Venom Allergen II	Solenopsis richteri (Black imported fire ant)	158
Sol r 3	VA3_SOLRI	P35779	Venom Allergen III	Solenopsis richteri (Black imported fire ant)	159
Ves c 5	VA51_VESCR	P35781	Venom Allergen 5.01	Vespa crabro	160

				(European hornet)	
Ves c 5	VA52_VESCR	P35782	Venom Allergen 5.02	Vespa crabro (European hornet)	161
Ves f 5	VA5_VESFL	P35783	Venom Allergen 5	Vespula flavopilosa (Yellow jacket) (Wasp)	162
Ves g 5	VA5_VESGE	P35784	Venom Allergen 5	Vespula germanica (Yellow jacket) (Wasp)	163
Ves m 1	PA1_VESMC	P51528	Phospholipase A1	Vespula maculifrons (Eastern yellow jacket) (Wasp)	164
Ves m 5	VA5_VESMC	P35760	Venom Allergen 5	Vespula maculifrons (Eastern yellow jacket) (Wasp)	165
Ves p 5	VA5_VESPE	P35785	Venom Allergen 5	Vespula pensylvanica (Western yellow jacket) (Wasp)	166
Ves s 5	VA5_VESSQ	P35786	Venom Allergen 5	Vespula squamosa (Southern yellow jacket) (Wasp)	167
Ves v 1	PA1_VESVU	P49369	Phospholipase A1 [Precursor]	Vespula vulgaris (Yellow jacket) (Wasp)	168
Ves v 2	HUGA_VESVU	P49370	Hyaluronoglucosami nidase	Vespula vulgaris (Yellow jacket) (Wasp)	169
Ves v 5	VA5_VESVU	Q05110	Venom Allergen 5 [Precursor]	Vespula vulgaris (Yellow jacket) (Wasp)	170
Ves vi 5	VA5_VESVI	P35787	Venom Allergen 5	Vespula vidua (Yellow jacket) (Wasp)	171
Vesp m 5	VA5_VESMA	P81657	Venom Allergen 5	Vespa mandarinia (Hornet)	172
Zea m 1	MPZ1_MAIZE	Q07154	Pollen Allergen Zea m 1	Zea mays (Maize)	173

Suitable vectors are prepared using standard techniques of recombinant DNA technology, and are, for example, described in "Molecular Cloning: A Laboratory Manual", 2nd edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology", 4th edition (D.M. Weir & C.C. Blackwell, eds., Blackwell Science Inc., 1987); "Gene Transfer Vectors for Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994); and "Current Protocols in Immunology" (J.E. Coligan et al., eds., 1991). Isolated plasmids and DNA fragments are cleaved, tailored, and ligated together in a specific order to generate the desired vectors. After ligation, the vector containing the gene to be expressed is transformed into a suitable host cell.

Host cells can be any eukaryotic or prokaryotic hosts known for expression of heterologous proteins. Accordingly, the polypeptides of the present invention can be expressed in eukaryotic hosts, such as eukaryotic microbes (yeast) or cells isolated from multicellular organisms (mammalian cell cultures), plants and insect cells. Examples of mammalian cell lines suitable for the expression of heterologous polypeptides include monkey kidney CV1 cell line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney cell line 293S (Graham *et al*, J. Gen. Virol. 36:59 [1977]); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary (CHO) cells (Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216 [1980]; monkey kidney cells (CVI-76, ATCC CCL 70); African green monkey cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); human lung cells (W138, ATCC CCL 75); and human liver cells (Hep G2, HB 8065). In general myeloma cells, in particular those not producing any endogenous antibody, e.g. the non-immunoglobulin producing myeloma cell line SP2/0, are preferred for the production of the fusion molecules herein.

Eukaryotic expression systems employing insect cell hosts may rely on either plasmid or baculoviral expression systems. The typical insect host cells are derived from the fall army worm (*Spodoptera frugiperda*). For expression of a foreign protein these cells are infected with a recombinant form of the baculovirus *Autographa californica* nuclear polyhedrosis virus which has the gene of interest expressed under the control of the viral polyhedrin promoter. Other insects infected by this virus include a cell line known commercially as "High 5" (Invitrogen) which is

derived from the cabbage looper (*Trichoplusia ni*). Another baculovirus sometimes used is the *Bombyx mori* nuclear polyhedrosis virus which infect the silk worm (*Bombyx mori*). Numerous baculovirus expression systems are commercially available, for example, from Invitrogen (Bac-N-Blue™), Clontech (BacPAK™ Baculovirus Expression System), Life Technologies (BAC-TO-BAC™), Novagen (Bac Vector System™), Pharmingen and Quantum Biotechnologies). Another insect cell host is common fruit fly, *Drosophila melanogaster*, for which a transient or stable plasmid based transfection kit is offered commercially by Invitrogen (The DES™ System).

Saccharomyces cerevisiae is the most commonly used among lower eukaryotic hosts. However, a number of other genera, species, and strains are also available and useful herein, such as *Pichia pastoris* (EP 183,070; Sreekrishna *et al.*, J. Basic Microbiol. 28:165-278 (1988)). Yeast expression systems are commercially available, and can be purchased, for example, from Invitrogen (San Diego, CA). Other yeasts suitable for bi-functional protein expression include, without limitation, *Kluyveromyces* hosts (U.S. Pat. No. 4,943,529), e.g. *Kluyveromyces lactis*; *Schizosaccharomyces pombe* (Beach and Nurse, Nature 290:140 (1981); *Aspergillus* hosts, e.g. *A. niger* (Kelly and Hynes, EMBO J. 4:475-479 (1985))] and *A. nidulans* (Ballance *et al.*, Biochem. Biophys. Res. Commun. 112:284-289 (1983)), and *Hansenula* hosts, e.g. *Hansenula polymorpha*. Yeasts rapidly growth on inexpensive (minimal) media, the recombinant can be easily selected by complementation, expressed proteins can be specifically engineered for cytoplasmic localization or for extracellular export, and are well suited for large-scale fermentation.

Prokaryotes are the preferred hosts for the initial cloning steps, and are particularly useful for rapid production of large amounts of DNA, for production of single-stranded DNA templates used for site-directed mutagenesis, for screening many mutants simultaneously, and for DNA sequencing of the mutants generated. *E. coli* strains suitable for the production of the peptides of the present invention include, for example, BL21 carrying an inducible T7 RNA polymerase gene (Studier *et al.*, Methods Enzymol. 185:60-98 (1990)); AD494 (DE3); EB105; and CB (*E. coli* B) and their derivatives; K12 strain 214 (ATCC 31,446); W3110 (ATCC 27,325); X1776 (ATCC 31,537); HB101 (ATCC 33,694); JM101 (ATCC 33,876); NM522 (ATCC 47,000); NM538 (ATCC 35,638); NM539 (ATCC 35,639), etc. Many other species and genera of prokaryotes may be used as well. Indeed, the peptides of the present invention can be readily produced in large amounts by utilizing recombinant protein expression in bacteria, where the peptide is fused to a cleavable ligand used for affinity purification.

Suitable promoters, vectors and other components for expression in various host cells are well known in the art and are disclosed, for example, in the textbooks listed above.

Whether a particular cell or cell line is suitable for the production of the polypeptides herein in a functionally active form, can be determined by empirical analysis. For example, an expression construct comprising the coding sequence of the desired molecule may be used to transfect a candidate cell line. The transfected cells are then grown in culture, the medium collected, and assayed for the presence of secreted polypeptide. The product can then be quantitated by methods known in the art, such as by ELISA with an antibody specifically binding the IgG, IgE, or allergen portion of the molecule.

In certain instances, especially if the two polypeptide sequences making up the bifunctional molecule of the present invention are connected with a non-polypeptide linker, it may be advantageous to individually synthesize the first and second polypeptide sequences, e.g. by any of the recombinant approaches discussed above, followed by functionally linking the two sequences.

Alternatively, the two polypeptide sequences, or the entire molecule, may be prepared by chemical synthesis, such as solid phase peptide synthesis. Such methods are well known to those skilled in the art. In general, these methods employ either solid or solution phase synthesis methods, described in basic textbooks, such as, for example, J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, 2nd Ed., Pierce Chemical Co., Rockford, Ill. (1984) and G. Barany and R. B. Merrifield, *The Peptide: Analysis Synthesis, Biology*, editors E. Gross and J. Meienhofer, Vol. 2, Academic Press, New York, (1980), pp. 3-254, for solid phase peptide synthesis techniques; and M. Bodansky, *Principles of Peptide Synthesis*, Springer-Verlag, Berlin (1984) and E. Gross and J. Meienhofer, Eds., *The Peptides: Analysis, Synthesis, Biology*, *supra*, Vol. 1, for classical solution synthesis.

The fusion molecules of the present invention may include amino acid sequence variants of native immunoglobulin (e.g. IgG and/or IgE) or allergen (e.g., Ara h 2 sequences). Such amino acid sequence variants can be produced by expressing the underlying DNA sequence in a suitable recombinant host cell, or by *in vitro* synthesis of the desired polypeptide, as discussed above. The nucleic acid sequence encoding a polypeptide variant is preferably prepared by site-directed mutagenesis of the nucleic acid sequence encoding the corresponding native (e.g. human) polypeptide. Particularly preferred is site-directed mutagenesis using polymerase chain reaction (PCR) amplification (see, for example, U.S. Pat. No. 4,683,195 issued 28 July 1987; and Current

Protocols In Molecular Biology, Chapter 15 (Ausubel *et al.*, ed., 1991). Other site-directed mutagenesis techniques are also well known in the art and are described, for example, in the following publications: Current Protocols In Molecular Biology, *supra*, Chapter 8; Molecular Cloning: A Laboratory Manual, 2nd edition (Sambrook *et al.*, 1989); Zoller *et al.*, Methods Enzymol. 100:468-500 (1983); Zoller & Smith, DNA 3:479-488 (1984); Zoller *et al.*, Nucl. Acids Res., 10:6487 (1987); Brake *et al.*, Proc. Natl. Acad. Sci. USA 81:4642-4646 (1984); Botstein *et al.*, Science 229:1193 (1985); Kunkel *et al.*, Methods Enzymol. 154:367-82 (1987), Adelman *et al.*, DNA 2:183 (1983); and Carter *et al.*, Nucl. Acids Res., 13:4331 (1986). Cassette mutagenesis (Wells *et al.*, Gene, 34:315 [1985]), and restriction selection mutagenesis (Wells *et al.*, Philos. Trans. R. Soc. London SerA, 317:415 [1986]) may also be used.

Amino acid sequence variants with more than one amino acid substitution may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be mutated simultaneously, using one oligonucleotide that codes for all of the desired amino acid substitutions. If, however, the amino acids are located some distance from one another (e.g. separated by more than ten amino acids), it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed. In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions. The alternative method involves two or more rounds of mutagenesis to produce the desired mutant.

The polypeptides of the invention can also be prepared by the combinatorial peptide library method disclosed, for example, in International Patent Publication PCT WO 92/09300. This method is particularly suitable for preparing and analyzing a plurality of molecules, that are variants of a given predetermined sequences, and is, therefore, particularly useful in identifying polypeptides with improved biological properties, which can then be produced by any technique known in the art, including recombinant DNA technology and/or chemical synthesis.

3. Therapeutic uses of the fusion molecules of the invention

The present invention provides a new therapeutic strategy for treating immediate hypersensitivity diseases mediated through the high-affinity IgE receptor. In particular, the

invention provides compounds for use in the treatment of both allergic diseases where IgE bridging of FcεR receptors occurs and autoimmune disorders where autoantibodies bind to the FcεR.

Nature of the diseases targeted Following the Gell and Coombs Classification, allergic reactions are classified depending on the type of immune response induced and the resulting tissue damage that develops as a result of reactivity to an antigen. A Type I reaction (immediate hypersensitivity) occurs when an antigen (called an allergen in this case) entering the body encounters mast cells or basophils which are sensitized as a result of IgE to that antigen being attached to its high-affinity receptor, FcεRI. Upon reaching the sensitized mast cell, the allergen cross-links IgE bound to FcεRI, causing an increase in intracellular calcium (Ca^{2+}) that triggers the release of pre-formed mediators, such as histamine and proteases, and newly synthesized, lipid-derived mediators such as leukotrienes and prostaglandins. These autocooids produce the acute clinical symptoms of allergy. The stimulated basophils and mast cells will also produce and release proinflammatory mediators, which participate in the acute and delayed phase of allergic reactions.

As discussed before and shown in Table 1 above, a large variety of allergens has been identified so far, and new allergens are identified, cloned and sequenced practically every day.

Ingestion of an allergen results in gastrointestinal and systemic allergic reactions. The most common food allergens involved are peanuts, shellfish, milk, fish, soy, wheat, egg and tree nuts such as walnuts. In susceptible people, these foods can trigger a variety of allergic symptoms, such as nausea, vomiting, diarrhea, urticaria, angioedema, asthma and full-blown anaphylaxis.

Inhalation of airborne allergens results in allergic rhinitis and allergic asthma, which can be acute or chronic depending on the nature of the exposure(s). Exposure to airborne allergens in the eye results in allergic conjunctivitis. Common airborne allergens includes pollens, mold spores, dust mites and other insect proteins. Grass and weed and tree pollens are the most common cause of seasonal hay fever and allergic asthma.

Cutaneous exposure to an allergen, e.g. natural rubber latex proteins as found in latex gloves, may result in local allergic reactions manifest as hives (urticaria) at the places of contact with the allergen.

Systemic exposure to an allergen such as occurs with a bee sting, the injection of penicillin, or the use of natural rubber latex (NRL) gloves inside a patient during surgery may result in, cutaneous, gastrointestinal and respiratory reactions up to and including airway obstruction and full blown anaphylaxis. Hymenoptera stings are insects that commonly cause allergic reactions, often

leading the anaphylactic shock. Examples include various bees including honeybees, yellow jackets, yellow hornets, wasps and white-faced hornets. Certain ants known as fire ants (*Solenopsis invicta*) are an increasing cause of allergy in the US as they expand their range in this country. Proteins in NRL gloves have become an increasing concern to health care workers and patients and at present, there is no successful form of therapy for this problem except avoidance.

Uses of compounds for targeted diseases The compounds disclosed herein can be used to acute or chronically inhibit IgE mediated reaction to major environmental and occupational allergens, can be used to provide for allergy vaccination (immunotherapy) to induce a state of non-allergic reactivity to specific allergens and can also have a prophylactic effect against allergic disease by preventing allergic sensitization to environmental and occupational allergens when administered to at-risk individuals (e.g., those at genetic risk of asthma and those exposed to occupational allergens in the workplace).

The bifunctional gamma-epsilon compounds described can be used to prevent allergic reactions to any specific allergen or group of allergens. By occupying a critical number of FcεRI receptors, these molecules will inhibit the ability of basophils and mast cells to react to any allergen so as to prevent including, without limitation, asthma, allergic rhinitis, atopic dermatitis, food allergies, urticaria and angioedema, up to and including anaphylactic shock. Thus these compounds could be used acutely to desensitize a patient so that the administration of a therapeutic agent (e.g. penicillin) can be given safely. Similarly, they can be used to desensitize a patient so that standard allergen vaccination may be given with greater safety, e.g. peanut or latex treatment. They can also be used as chronic therapy to prevent clinical reactivity to prevent environmental allergens such as foods or inhalant allergens.

The present invention as gamma allergen bifunctional fusion molecules provides for a novel form of allergy vaccination that will be safer and more effective the treatment of a varieties of IgE mediated allergic reactivity, including, without limitation, asthma, allergic rhinitis, atopic dermatitis, food allergies, urticaria and angioedema, up to and including anaphylactic shock. Having the allergen fused to a molecule that will bind to FcγRIIb on mast cells basophils will prevent the allergen being able to induce local or systemic allergic reactions. Such local or systemic allergic reactions are major problem in allergen vaccination as currently practiced. The gamma-allergen fusion proteins will be able to be given in higher doses over a shorter interval and with greater safety than standard allergen therapy. In addition, use of the gamma-allergen compounds will cause

antigen specific desensitization to that specific allergen. Thus the gamma-allergen compounds will give a window of safe exposure to the allergen be it as an acute or recurring treatment as would be needed in using a therapeutic monoclonal antibody to which a patient has developed an allergic (IgE) response or as chronic treatment for prevention of unintentional exposures such as occurs with peanut allergens. This use is expected gain added importance, as the number of recombinant biological products entering the clinical arena will be increasing dramatically in the near future. The gamma-allergen compounds can even be used along with conventional allergen vaccination so as to provide an extra margin of safety while large doses of standard allergen are given.

In addition, the chimeric gamma-epsilon compounds herein hold great promise for the treatment of chronic urticaria and angioedema. Urticaria is a skin symptom that may accompany allergies but often is idiopathic. It is a relatively common disorder caused by localized cutaneous mast cell degranulation, with resultant increased dermal vascular permeability culminating in pruritic wheals. Angioedema is a vascular reaction involving the deep dermis or subcutaneous or submucosal tissues caused by localized mast cell degranulation. This results in tissue swelling that is pruritic or painful. Chronic urticaria and angioedema often occur together although they occur individually as well. These conditions are common and once present for more than six months, they often last a decade or more. Although not fatal, they are very troubling to patients as the frequent recurring attacking disrupt daily activities and thereby result in significant morbidity. Standard therapy is often unsuccessful in these conditions and they are distressing to the point that chemotherapy with cyclosporine and other potent immunosuppressive drugs has recently been advocated. Increasing evidence suggests that as many as 60% of patients with these conditions actually have an autoimmune disease, in which they make functional antibodies against the FcεRI receptor. For further details, see Hide *et al.*, *N. Engl. J. Med.* 328:1599-1604 (1993); Fiebiger *et al.*, *J. Clin. Invest.* 96:2606-12 (1995); Fiebiger *et al.*, *J. Clin. Invest.* 101:243-51 (1998); Kaplan, A.P., Urticaria and Angioedema, In: Inflammation: Basic Principles and Clinical Correlates (Gallin and Snyderman eds.), 3rd Edition, Lippincott & Wilkins, Philadelphia, 1999, pp. 915-928. The fusion molecules of the present invention are believed to form the basis for a novel and effective treatment of these diseases by safely blocking access to the FcεRI.

For therapeutic uses, including prevention, the compounds of the invention can be formulated as pharmaceutical compositions in admixture with pharmaceutically acceptable carriers or diluents. Methods for making pharmaceutical formulations are well known in the art.

Techniques and formulations generally may be found in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Co., Easton, PA 1990. See, also, Wang and Hanson "Parenteral Formulations of Proteins and Peptides: Stability and Stabilizers", *Journal of Parenteral Science and Technology*, Technical Report No. 10, Supp. 42-2S (1988). A suitable administration format can best be determined by a medical practitioner for each patient individually.

Pharmaceutical compositions of the present invention can comprise a fusion molecule of the present invention along with conventional carriers and optionally other ingredients.

Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, inhalation, or by injection. Such forms should allow the agent or composition to reach a target cell whether the target cell is present in a multicellular host or in culture. For example, pharmacological agents or compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms that prevent the agent or composition from exerting its effect.

Carriers or excipients can also be used to facilitate administration of the compound. Examples of carriers and excipients include calcium carbonate, calcium phosphate, various sugars such as lactose, glucose, or sucrose, or types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols and physiologically compatible solvents. The compositions or pharmaceutical composition can be administered by different routes including, but not limited to, oral, intravenous, intra-arterial, intraperitoneal, subcutaneous, intranasal or intrapulmonary routes.

The desired isotonicity of the compositions can be accomplished using sodium chloride or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol, polyols (such as mannitol and sorbitol), or other inorganic or organic solutes.

For systemic administration, injection is preferred, *e.g.*, intramuscular, intravenous, intra-arterial, etc. For injection, the compounds of the invention are formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. Alternatively, the compounds of the invention are formulated in one or more excipients (*e.g.*, propylene glycol) that are generally accepted as safe as defined by USP standards. They can, for example, be suspended in an inert oil, suitably a vegetable oil such as sesame, peanut, olive oil, or other acceptable carrier. Preferably, they are suspended in an aqueous carrier, for example, in an isotonic buffer solution at pH of about 5.6 to 7.4. These compositions can be sterilized by conventional sterilization techniques, or can be sterile filtered. The compositions can contain

pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH buffering agents. Useful buffers include for example, sodium acetate/acetic acid buffers. A form of repository or "depot" slow release preparation can be used so that therapeutically effective amounts of the preparation are delivered into the bloodstream over many hours or days following transdermal injection or delivery. In addition, the compounds can be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

Alternatively, certain molecules identified in accordance with the present invention can be administered orally. For oral administration, the compounds are formulated into conventional oral dosage forms such as capsules, tablets and tonics.

Systemic administration can also be by transmucosal or transdermal. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, bile salts and fusidic acid derivatives. In addition, detergents can be used to facilitate permeation. Transmucosal administration can be, for example, through nasal sprays or using suppositories.

A preferred route for administration of the compounds of the invention may be inhalation for intranasal and/or intrapulmonary delivery. For administration by inhalation, usually inhalable dry power compositions or aerosol compositions are used, where the size of the particles or droplets is selected to ensure deposition of the active ingredient in the desired part of the respiratory tract, e.g. throat, upper respiratory tract or lungs. Inhalable compositions and devices for their administration are well known in the art. For example, devices for the delivery of aerosol medications for inspiration are known. One such device is a metered dose inhaler that delivers the same dosage of medication to the patient upon each actuation of the device. Metered dose inhalers typically include a canister containing a reservoir of medication and propellant under pressure and a fixed volume metered dose chamber. The canister is inserted into a receptacle in a body or base having a mouthpiece or nosepiece for delivering medication to the patient. The patient uses the device by manually pressing the canister into the body to close a filling valve and capture a metered dose of medication inside the chamber and to open a release valve which releases the captured, fixed volume of medication in the dose chamber to the atmosphere as an aerosol mist. Simultaneously, the patient inhales through the mouthpiece to entrain the mist into the airway. The patient then

releases the canister so that the release valve closes and the filling valve opens to refill the dose chamber for the next administration of medication. See, for example, U.S. Pat. No. 4,896,832 and a product available from 3M Healthcare known as Aerosol Sheathed Actuator and Cap.

Another device is the breath actuated metered dose inhaler that operates to provide automatically a metered dose in response to the patient's inspiratory effort. One style of breath actuated device releases a dose when the inspiratory effort moves a mechanical lever to trigger the release valve. Another style releases the dose when the detected flow rises above a preset threshold, as detected by a hot wire anemometer. See, for example, U.S. Pat. Nos. 3,187,748; 3,565,070; 3,814,297; 3,826,413; 4,592,348; 4,648,393; 4,803,978.

Devices also exist to deliver dry powdered drugs to the patient's airways (see, e.g. U.S. Patent No. 4,527,769) and to deliver an aerosol by heating a solid aerosol precursor material (see, e.g. U.S. Patent No. 4,922,901). These devices typically operate to deliver the drug during the early stages of the patient's inspiration by relying on the patient's inspiratory flow to draw the drug out of the reservoir into the airway or to actuate a heating element to vaporize the solid aerosol precursor.

Devices for controlling particle size of an aerosol are also known, see, for example, U.S. Patent Nos. 4,790,305; 4,926,852; 4,677,975; and 3,658,059.

For topical administration, the compounds of the invention are formulated into ointments, salves, gels, or creams, as is generally known in the art.

If desired, solutions of the above compositions can be thickened with a thickening agent such as methyl cellulose. They can be prepared in emulsified form, either water in oil or oil in water. Any of a wide variety of pharmaceutically acceptable emulsifying agents can be employed including, for example, acacia powder, a non-ionic surfactant (such as a Tween), or an ionic surfactant (such as alkali polyether alcohol sulfates or sulfonates, e.g., a Triton).

Compositions useful in the invention are prepared by mixing the ingredients following generally accepted procedures. For example, the selected components can be mixed simply in a blender or other standard device to produce a concentrated mixture which can then be adjusted to the final concentration and viscosity by the addition of water or thickening agent and possibly a buffer to control pH or an additional solute to control tonicity.

The amounts of various compounds for use in the methods of the invention to be administered can be determined by standard procedures. Generally, a therapeutically effective

amount is between about 100 mg/kg and 10^{-12} mg/kg depending on the age and size of the patient, and the disease or disorder associated with the patient. Generally, it is an amount between about 0.05 and 50 mg/kg, more preferably between about 1.0 and 10 mg/kg for the individual to be treated. The determination of the actual dose is well within the skill of an ordinary physician.

5 The compounds of the present invention may be administered in combination with one or more further therapeutic agent for the treatment of IgE-mediated allergic diseases or conditions. Such further therapeutic agents include, without limitation, corticosteroids, β -antagonists, theophylline, leukotriene inhibitors, allergen vaccination, soluble recombinant human soluble IL-4 receptors (Immunogen), anti-IL-4 monoclonal antibodies (Protein Design Labs), and anti-IgE
10 antibodies, such as the recombinant human anti-IgE monoclonal antibody rhuMAb-E25 (Genentech, Inc.) which is currently in advanced clinical trials for the treatment of patients with atopic asthma, and other allergic diseases, such as allergic rhinitis and atopic dermatitis (see, e.g. Barnes, *The New England Journal of Medicine* 341:2006-2008 (1999)). Thus the compounds of the present invention can be used to supplement traditional allergy therapy, such as corticosteroid therapy performed with
15 inhaled or oral corticosteroids.

4. Articles of manufacture

20 The invention also provides articles of manufacture comprising the single-chain fusion compounds herein. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The container may also be an inhalation
25 device such as those discussed above. At least one active agent in the composition is a fusion compound of the invention. The label or package insert indicates that the composition is used for treating the condition of choice, such as an allergic condition, e.g. asthma or any of the IgE-mediated allergies discussed above. The article of manufacture may further comprise a further container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection
30 (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include

other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

Further details of the invention are illustrated by the following non-limiting Example.

Example

Construction and Expression of a Chimeric Human Fc γ -Fc ϵ Fusion Protein

Materials and Methods

Plasmids, vectors and cells - Plasmid pAG 4447 containing genomic DNA encoding human IgE constant region and expression vector pAN 1872 containing human genomic DNA encoding the hinge-CH2-CH3 portion of IgG₁ constant region were obtained from the laboratory of Dr. Morrison. pAN 1872 is derived from the pDisplay vector (Invitrogen). pAG 4447 was developed and used as a cloning intermediate in the construction of a human IgE expression vector disclosed in *J. Biol. Chem.* 271:3428-3436 (1996). To construct the chimeric gene, a pair of primers were designed to amplify the human IgE constant region (CH2-CH3-CH4).

5'-end primer:

5'GCTCGAGGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGTTCAC
CCCGCCACCGTGAAG3' (SEQ ID NO: 174),

containing a flexible linker sequence and an XhoI site.

3' end primer:

5'GGCGGCCGCTCATTTACCGGGATTACAGACAC3' (SEQ ID NO: 175),

containing an NotI.

After amplification, the PCR products were cloned into pCR2.1 vector (Invitrogen). The sequences of the products were confirmed. Then, the XhoI-NotI fragment was inserted into the 1782 pAN vector, following the IgG₁ CH3 domain in the same reading frame by a (Gly₄Ser)₃ flexible linker. SP2.0 murine myeloma cell line was selected as host for expression because it does not secrete any antibody.

Expression and Purification - The expression vector containing chimeric Fc γ -Fc ϵ gene was linearized at the PcuI site and transfected into SP2/0 cells by electroporation (Bio-Rad). Stable transfectants were selected for growth in medium containing 1 mg/ml geneticine. Clones producing the fusion protein were identified by ELISA using plates coating anti-human IgE (CIA7.12) or IgG (Sigma) antibody. Supernatants from clones were added to wells, and bound protein was detected

using goat anti-human IgE or IgG conjugated to alkaline phosphatase (KPL). The fusion protein was purified from the supernatants and ascites by using rProtein A column (Pharmacia).

Western Blotting - The purified protein was run on 7.5% SDS polyacrylamide gel. After transfer, the nylon membrane was blocked by 4% bovine serum albumin/PBS/Tween overnight at 4 °C. For protein detection, the blot was probed with either goat anti-human IgE (ε chain specific) or goat anti-human IgG (γ chain-specific) conjugated to alkaline phosphatase (KPL). Color development was performed with an alkaline phosphatase conjugated substrate kit (Bio-Rad).

Binding Test - In order to confirm the binding, FcεRI transfected cells (CHO 3D10) or human HMC-1 cells that express FcγRIIb but not FcεRI were stained with purified fusion protein and then analyzed by flow cytometry. Briefly, cells were collected and washed. The cells were then incubated with 5 μl of 1 mg/ml GE2, PS IgE or human IgG at 4 °C for 60 minutes. After two washes, the cells were stained with FITC conjugated anti-human IgE or IgG at 4 °C for 60 minutes, and visualized by flow cytometry.

Inhibition of Basophil Histamine Release - Acid-stripped Percoll-enriched human blood basophils were primed with 1-10 μg/ml of chimeric human anti-NP IgE at 37 °C in a 5% CO₂ incubator and one hour later, challenged with 30 ng of NP-BSA (Kepley, *J. Allergy Clin. Immunol.* 106:337-348(2000)). Histamine release was measured in the supernatants 30 minutes later. GE2 or control human myeloma IgE was added at various doses and times to test the effects on histamine release.

Passive Cutaneous Anaphylaxis Model - Transgenic mice expressing the human FcεRIα chain and with the murine FcεRIα chain knocked out (provided by Dr. Jean-Pierre Kinet, Harvard Medical School, Boston, MA, Dombrowicz, *et al*, *J. Immunol.* 157:1645-1654. (1996)) were primed cutaneously with either recombinant human anti-dansyl or anti-NP IgE. Individual sites were then injected with saline, GE2 or IgE myeloma protein. Four hours later, mice were given a systemic challenge with dansyl-OVA or NP-BSA plus Evans blue, and the resulting area of reaction was measured.

Results

Western blotting showed that the chimeric protein (designated GE2) was expressed as the predicted dimer of approximately 140 kD. The GE2 protein reacted with both anti-human ε and anti-human γ chain-specific antibodies.

GE2 showed the ability to inhibit IgE-mediated release of histamine from fresh human basophils. The results of the dose-dependent inhibition of basophil histamine release using the fusion protein GE2 (\pm SEM; n+3 separate donors, each in duplicate) are shown in Figure 8. The data show that, when added to fresh human basophils along with the sensitizing anti-NP IgE antibody, GE2 inhibited subsequent NP-induced release of histamine in a dose-dependent manner, more effectively than an equivalent amount of native human IgE protein. This was time dependent as expected with the greatest effect being observed when the GE2 was added with the sensitizing anti-NP IgE antibody. No effect was observed if the GE2 was given simultaneously with the antigen challenge.

To test the *in vivo* function of GE2, the transgenic passive cutaneous anaphylaxis described above was used. The results are shown in Figure 9. The size and color of the reaction at the sites of GE2 injection were decreased compared to those injected with comparable amount of human IgE. These results demonstrate that the GE2 protein is able to inhibit mast cell/basophil function greater than an equivalent amount of IgE and implicates binding to both Fc ϵ RI and FC γ R..

Analysis of binding using flow cytometry showed that the GE2 protein bound in a fashion similar to native IgE to the human Fc γ RII expressed on HMC-1 cells. The data are shown in Figure 10. Similar results were obtained for the Fc ϵ RI on 3D10 cells, as shown in Figure 11.

All references cited throughout the specification are hereby expressly incorporated by reference. It is understood that the application of the teachings of the present invention to a specific problem or situation will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and representative processes for their production and use should not be construed to limit the invention.